EepR Mediates Secreted-Protein Production, Desiccation Survival, and Proliferation in a Corneal Infection Model

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Serratia marcescens is a soil- and water-derived bacterium that secretes several host-directed factors and causes hospital infections and community-acquired ocular infections. The putative two-component regulatory system composed of EepR and EepS regulates hemolysis and swarming motility through transcriptional control of the *swrW* gene and pigment production through control of the *pigA-pigN* operon. Here, we identify and characterize a role for EepR in regulation of exoenzyme production, stress survival, cytotoxicity to human epithelial cells, and virulence. Genetic analysis supports the model that EepR is in a common pathway with the widely conserved cyclic-AMP receptor protein that regulates protease production. Together, these data introduce a novel regulator of host-pathogen interactions and secreted-protein production.

Secreted enzymes such as proteases can act as immune system and physiology modulators and virulence factors during infection by a wide variety of pathogens (1–5). *Serratia marcescens* is a Gram-negative coccobacillus bacterium isolated from a variety of environmental niches and the human gut. It is an important opportunistic pathogen in hospitals (6–9), and isolates collected in intensive care units became more resistant to antibiotics between 2004 and 2009 (10). *S. marcescens* is also a leading contaminant of contact lens cases and contact lenses and can cause vision-threatening microbial keratitis and other ocular infections (11–13). *S. marcescens* synthesizes several secreted enzymes, including proteases, a potent nuclease, chitinases, and other enzymes that have been used for biotechnological applications or have been implicated in cytotoxicity to mammalian cells and pathogenesis (14–16). However, the regulation of these enzymes is poorly understood.

Known regulators of *S. marcescens* proteolysis include the *crp* gene that encodes the cyclic-AMP (cAMP) receptor protein CRP. Mutation of *crp* yields bacteria with elevated production of the protease serralyisin (PrtS; also known as PrtA) and a loss of secreted-chitinase and -lipase activities (17). Matsuyama and colleagues observed reduced expression of extracellular protease activity when the *hexS* gene, an *lhrA* homolog, was expressed from a multicopy plasmid, suggesting a negative regulatory role for this protein (63).

Extracellular proteases, such as the *S. marcescens* PrtS metalloprotease, are considered important virulence factors. *In vitro* studies indicate that serralyisin is cytotoxic to a variety of cell types (16, 21, 22). PrtS contributed to tissue damage in corneal infections using a rabbit model (23), altered immune cell interactions using a silkworm model (20, 24), and enhanced secondary infections by a respiratory virus in mice (25). Those studies provided the impetus to determine regulators of *S. marcescens* protease production.

A putative regulatory system that includes the response regulator-like protein EepR and a putative hybrid-histidine kinase, EepS, was recently identified for its role in controlling expression of swarming motility, pigmentation, and hemolysis (26). At the same time, *eepR* and *eepS* transposon mutants were found to be defective in secreted-protease production, but this was not explored in the previous study. The current study characterizes the role of EepR in exoenzyme production and the importance of

EepR in cytotoxicity and virulence. The data from this study support the idea that EepR is a novel regulator of secreted proteins and host-pathogen interactions.

MATERIALS AND METHODS

Microbial strains, media, and growth. *S. marcescens* strains are listed in Table 1, and plasmids are listed in Table 2. Bacteria were grown in LB medium (32) (0.5% yeast extract, 1% tryptone, 0.5% NaCl) with or without 1.5% agar. The *Escherichia coli* strains used were EC100D *pir-116* (Epicentre), SM10 *λpir*, and S17-1 *λpir* (33). Antibiotics used in this study included gentamicin (10 μg/ml), kanamycin at 100 μg/ml for *S. marcescens* and 50 μg/ml for *E. coli*, and tetracycline (10 μg/ml). A *pigA* deletion mutation was made in strain K904 by two-step allelic replacement using pMQ305 and was verified exactly as previously described (31).

Transcriptional analysis. RNA was prepared from cultures at optical densities at 600 nm (OD₆₀₀) of 1 and 3, and quantitative reverse transcriptase PCR (qPCR) was performed exactly as previously described (26). Primer sequences (all listed as 5' to 3') for transcript quantification were AACTGGAGGAAGGTGGGGAT and AGGAGGTGATCCAACCGCA for the 16S rRNA gene, AAAACTTCCCGTACCCTGCT and GTTCGGC TTGGTGATGAAAT for *chp21*, GGGGTAACGGGATTTCAGATT and TA ATCCGGGAAGGAGAAGGT for *prtS*, and GGCCTGTTCGACTACA GCTC and CTGTAGCCGGAGAAGTCCAG for *slpB*. Electrophoretic mobility shift assay (EMSA) analysis was performed with the same reagents as previously described (26). Primers to amplify the *prtS* promoter region were GGATTCATTCAATAATGAATAATGC and TACCTGATT GATATCAATCAGACAGATAGAC, and a 5'-biotinylated version of the latter oligonucleotide was ordered from Integrated DNA technologies. These amplify a 544-bp region, including 511 bp upstream of the *prtS* open reading frame. The amplicon was gel purified, and a portion was

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TABLE 1 *S. marcescens* strains used in this study

Strain	Description	Reference or source
CMS376	WT, wild-type strain, PIC3611	Presque Isle Cultures
CMS613	CMS376 with <i>crp-1</i> null mutation	27
CMS635	CMS376 with <i>swrW::Tn</i> mutation	19
CMS853	K904 clinical keratitis isolate	18
CMS1687	CMS376 with <i>crp-Δ4</i> mutation, Δ <i>crp</i>	18
CMS1787	Nima, pigmented strain	28
CMS2089	Nima Δ <i>eepR</i>	26
CMS2097	CMS376 Δ <i>eepR</i>	26
CMS2157	CMS376 <i>crp-Δ4 ΔeepR</i>	26
CMS2234	K904 <i>swrW::Tn</i>	29
CMS2862	CMS2904 with wild-type <i>eepR</i> (Db11) replacing Δ <i>eepR</i>	26
CMS2904	K904 Δ <i>eepR</i>	26
CMS2921	CMS2097 with wild-type <i>eepR</i> (Db11) replacing Δ <i>eepR</i>	26
CMS3207	K904 Δ <i>prtS</i>	22
CMS3982	K904 Δ <i>pigA</i>	This study

sequenced. An EMSA kit (Lightshift Chemiluminescent EMSA kit; Pierce) was used as specified by the manufacturer. Biotinylated target DNA (2 ng), purified maltose binding protein (MBP; 38 mM) or MBP-EepR (35 mM), and poly(dI-dC) (500 ng) as a nonspecific competitor were included in a 20-μl reaction mixture. An aliquot (10 μl) of the reaction mixture was separated on a polyacrylamide (5%) Tris-borate-EDTA (TBE) gel (Bio-Rad) with Tris-glycine running buffer. The EMSA was performed three times.

Protease assays. Milk agar plates (plates with brain heart infusion agar supplemented with skim milk at 1%) were used to test secreted-protease activity in colonies. Plates were incubated at 30°C for 24 to 48 h, and zones of clearing around colonies were visually assessed. Quantitative analysis was performed using azocasein (Sigma) as a colorimetric substrate (17, 34). Cultures were filtered to remove bacteria, and the filtrate (150 μl) was mixed with 250 μl of azocasein (2% [wt/vol]), subjected to vortex mixing, and placed at 37°C for 30 min. Trichloroacetic acid (TCA) (1.2 ml of a 0.6 N solution), was added to stop the reaction, and the mixture was incubated on ice for 15 min. Tubes were centrifuged for 10 min at 8,000 × g. Sodium hydroxide (1.4 ml of 1 N solution) was added to wells of a 24-well dish with 1.2-ml aliquots of the culture mixture. Protease-released azo dye was assessed by absorbance at 440 nm with a plate reader (Biotek Synergy 2), and this value was normalized by division by the optical density (OD₆₀₀) of the bacterial culture.

Polyacrylamide gel electrophoresis (PAGE) gels (8% to 16%) were used to separate TCA-precipitated secreted proteins obtained from filtered supernatants from cultures at an OD₆₀₀ of 2. Coomassie-stained gels were imaged with an Odyssey Li-Cor imager using the 700-nm channel. Pixel intensities from digital images were determined using ImageJ software (NIH). SlaA was identified from a gel slice by mass spectrometry at the University of Pittsburgh Proteomic Core Facility as previously described (35).

Chitinase assay. Assays were performed as previously described (17). Bacteria were grown in LB medium to saturation, diluted to an OD₆₀₀ of 2.0 with LB medium, and centrifuged for 5 min at 16,000 × g. Clarified supernatants (150 μl), or LB alone as a negative control, were mixed with 150 μl of acid-treated chitin azure (10 mg/ml) in sodium phosphate buffer (200 mM [pH 5.7]), and gentamicin was added to reach a concentration of 164 μg/ml to prevent bacterial growth. Samples were briefly subjected to vortex mixing and incubated for 72 h at 37°C with intermittent mixing. Tubes were centrifuged for 1 min at 16,000 × g, and the absorbance (A₅₆₀) was read from 150-μl aliquots of the supernatant with a plate reader (Biotek Synergy 2) to measure levels of chitinase-released dye. The experiment was performed on 2 days with a total of four biological replicates per genotype. Alternatively, 20 mg of acid-treated chitin azure was

TABLE 2 Plasmids used in this study

Plasmid	Description	Reference
pMQ118	Suicide vector <i>nptII</i> , <i>rpsL</i> , oriR6K	30
pMQ125	<i>P</i> _{BAD} - <i>lacZa</i> , oriPRO1600, oriP15a	30
pMQ131	pBBR1-based shuttle vector, <i>aphA-3</i>	30
pMQ132	pBBR1-based shuttle vector, <i>aacC1</i>	30
pMQ178	pMQ118 with <i>eepR</i> internal fragment	26
pMQ305	<i>pigA</i> allelic replacement vector	31
pMQ356	pMQ125 + His ₇ - <i>prtS</i>	22
pMQ364	pMQ131 + <i>eepR</i>	26
pMQ369	pMQ132 + <i>eepR</i> -His ₈	26

added to 25 ml of LB agar to generate chitin azure plates. Bacteria from overnight cultures (5 μl) were spotted onto the chitin azure plates and incubated at 30°C, and zones of clearing were imaged after 2 days (K904) or 7 days (CMS376).

2D-DIGE. Proteomic analysis of secreted proteins was performed as previously described (17). Briefly, 10 single colonies per genotype were grown in 10 separate test tubes in 5 ml of LB medium and aerated on a TC-7 tissue culture roller (New Brunswick Inc.) at a speed setting of 8 (62 rpm) at 30°C. After 24 h, the cultures were combined (separately for each genotype), culture turbidity (OD₆₀₀) was measured, and the culture density was adjusted to an OD₆₀₀ of 2.0 using LB. Bacteria were removed from 40-ml aliquots by centrifugation (10,000 × g for 10 min) followed by passage of the supernatant through a 0.22-μm-pore-size filter (Millex GV; Millipore). Bovine serum albumen (BSA) (5 μg/ml) was added to the wild-type (WT) strain (CMS376) and Δ *eepR* mutant (CMS2097) secreted fractions to serve as an internal loading control. Frozen filtered supernatants were sent to Applied Biomics (Hayward, CA) for two-dimensional difference gel electrophoresis (2D-DIGE) analysis and protein identification, as previously described (36). DeCyder 2D software was used to determine protein spots and ratios, and a subset of secreted proteins were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. The experiment was performed twice.

Cytotoxicity analysis. Human corneal limbal epithelial (HCLE) cells (37) were used for cytotoxicity assays with alamarBlue or Presto Blue as a viability dye as previously reported (38). HCLE cells were grown in keratinocyte serum-free medium (KSFM) (Gibco catalog no. 10724-011) containing 0.2 ng/ml embryonic growth factor (EGF) and 25 μg/ml bovine pituitary extract (BPE) to confluent monolayers in 24-well dishes. Monolayers were exposed to filtered bacterial supernatants or bacteria at a multiplicity of infection (MOI) as noted. For cytotoxicity assays in which bacteria were used to challenge HCLE cells, bacteria were grown in LB medium for 18 h with aeration, adjusted to an OD₆₀₀ of 1, washed with phosphate-buffered saline (PBS), and suspended in KSFM at the indicated MOI. HCLE cell layers were incubated with bacteria at 37°C and 5% CO₂ for 2 h. Bacteria and media were removed, cell layers were washed 3 times with warm PBS, and resazurin was used as a vitality stain (alamarBlue or Presto Blue) as suggested by the manufacturer (Invitrogen, Camarillo, CA). Cell layers were stained for 60 min, and vitality was measured by fluorescence using a plate reader (Biotek Synergy 2) with a 500-nm/27-nm excitation filter and a 620-nm/40-nm emission filter.

For determining the cytotoxicity of bacterial secretomes, bacteria were grown as described above and adjusted to an OD₆₀₀ of 2.0 with fresh LB broth. Bacteria were removed by centrifugation followed by filtration (Millex GV; Millipore) (0.22 μm pore size) of the supernatant. Monolayers were covered with KSFM (300 μl) and challenged with 200 μl of filtered supernatants and incubated for 4 h at 37°C and 5% CO₂. As noted above, the viability staining was measured by fluorescence.

Inflammatory marker array and IL-1β ELISA. Stratified HCLE cells grown in 12-well plates (Costar catalog no. 3513) were washed 3 times with PBS. Stratification medium (1 ml) was added to each well followed by 500 μl of bacterial secretomes, prepared as described above, and 500 μl LB medium was added as a mock control. After 24 h of incubation at 37°C

and 5% CO₂, supernatants from each group were collected and tested for inflammatory markers using a human Proteome Profiler cytokine array (R&D Systems, Minneapolis, MN; product ARY005). The cytokine array methodology includes mixing the cell culture supernatant with biotinylated antibodies specific for detection of 24 inflammatory markers according to the instructions provided by the manufacturer. Following this incubation, the mixture is exposed to a membrane with immobilized antibodies for the inflammatory markers in discrete spots on the membrane. After washing steps, streptavidin linked to horseradish peroxidase bound to the biotinylated antibody and chemiluminescent detection was performed. X-ray film was scanned, and the pixel densities of spots were determined and normalized to those of positive-control spots on each membrane using ImageJ software (NIH). For a more quantitative analysis of interleukin-1 beta (IL-1 β) levels, enzyme-linked immunosorbent assay (ELISA) analysis was performed using samples prepared as described for the inflammatory marker array, and IL-1 β was measured using a human IL-1 β ELISA kit (R&D Systems; DLB50). The experiment was performed three times with independent samples.

Rabbit keratitis infection model. New Zealand White (NZW) rabbits obtained from Harlan (Indianapolis, IN) were intrastromally injected in one eye with ~100 CFU of bacteria in 25 μ l using a 30-gauge needle. Bacteria were grown overnight in LB broth, washed in PBS, and diluted to the appropriate density. Infectious doses were verified by determining bacterial counts from aliquots of the inoculum. Four rabbits were injected for each of the two bacterial genotypes, and four were injected with PBS as a negative control. The experiment was repeated twice. After 48 h, the eyes were graded for clinical signs of infection and inflammation by masked observation by an experienced observer using a slit lamp. Signs were graded with a severity scale of 0 to 3 points, with a maximum cumulative score of 18 points, for the following criteria: conjunctivitis, chemosis, discharge, iritis, corneal edema, and corneal infiltrate. Immediately after clinical evaluation, a subset of animals was euthanized, corneal buttons (9.5 mm in diameter) were removed with a trephine, suspended in 1 ml PBS, and homogenized, and bacterial colony counts were determined. All experiments conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Stress survival analysis. Desiccation survival frequency was determined as follows. Cultures were grown overnight (~18 h) in LB medium with aeration, and the CFU numbers of the cultures were determined by dilution plating. A 10- μ l volume of each culture was placed in the bottom of a 24-well dish and dried in a laminar flow hood for 30 min, and the plates were incubated at 30°C. After 24 h, 1 ml of sterile PBS was added to each well and the bacteria were suspended by low-power sonication (QSonica Model Q55) (power level of 3 for two 30-s pulses) and enumerated by dilution plating on LB agar plates.

As a second level of desiccation analysis, bacteria were grown overnight in LB broth, adjusted to an OD₆₀₀ of 1, spun down, and suspended in PBS. A 20- μ l volume of the sample or serial dilution was placed in a 96-well dish, and the plates were dried in a laminar flow hood and then incubated at 30°C. After 24 h of desiccation challenge, 100 μ l of LB broth was added to each well and the reaction mixture was placed in a bag at 30°C with a wet paper towel. After 24 h, the optical density (OD₆₀₀) of each well was read with a plate reader.

Hydrogen peroxide resistance was determined by plating bacteria from overnight cultures, adjusted to an OD₆₀₀ of 0.05, on LB agar plates. A paper disc was added to the surface of the plate, 10 μ l of H₂O₂ (30%) was added to the disc, and the plate was incubated at 30°C. After 18 h, the zone of growth inhibition was measured with a ruler to a precision of 0.5 mm.

Heat tolerance was tested with bacteria from overnight cultures that were washed with PBS and normalized in PBS (1 ml) to an OD₆₀₀ of 2 in microcentrifuge tubes. CFU from the tubes were quantified by dilution plating, and the tubes were placed on a heating block set to 65°C. At various time points, samples were taken and CFU levels were determined

FIG 1 EepR is necessary for secreted-protease activity. (A) Secreted-protease activity measured from normalized stationary-phase supernatants using azocasein as a proteolytic substrate. Mutation of *eepR* severely reduces secreted-protease activity and suppresses the *crp* mutation phenotype. Means and standard deviations are shown ($n \geq 5$). WT, CMS376; *crp*, CMS613; *eepR*, CMS2097; *crp eepR*, CMS2157. (B) Complementation of the $\Delta eepR$ mutant protease defect. Means ($n = 7$) and standard deviations are shown. (C) Mutation of *eepR* in clinical isolate K904 reduces secreted-protease activity, and the defect can be complemented by *eepR* on a plasmid (pMQ369) but not by the vector pMQ132. K904, CMS853; K904 $\Delta eepR$, CMS2904. Means ($n = 8$) and standard deviations are shown. For all charts, asterisks indicate statistically significant differences from the WT, WT with vector, or K904 with vector results by ANOVA with Tukey's posttest ($P < 0.001$).

on LB agar plates. Six independent cultures were used for each genotype over two experiments.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software and one-way analysis of variance (ANOVA) with Tukey's posttest, Kruskal-Wallis, and Mann-Whitney U-tests, and two-tailed Student's *t* tests were used with significance set at a *P* value of <0.05 .

RESULTS

EepR mediates production of serralyisin and SlpB. The role of EepR in protease regulation was tested with two different strains of *S. marcescens*. Two strains were used because the importance of a gene in a given phenotype can vary between species and even between strains (39). One was a laboratory strain (CMS376) producing protease at a low level and the other a clinical isolate (K904) producing protease at a high level (22). The $\Delta eepR$ mutant (CMS2097) was significantly reduced in secreted-protease activity compared to the isogenic wild-type (WT) strain (CMS376) (Fig. 1A). The *eepR* mutant protease-defective phenotype was complemented by addition of *eepR* on a plasmid, demonstrating EepR's requirement in promoting protease production (Fig. 1B).

A keratitis clinical isolate, strain K904, produced more secreted-protease activity than the CMS376 laboratory strain (Fig. 1C ver-

FIG 2 EepR is required for production of wild-type levels of protease. (A and C) Expression of *prtS* (A) and *slpB* (C) in cultures was measured by qPCR at an OD₆₀₀ of 3. The values are expressed relative to 16S expression and were quantified using the $2^{-\Delta\Delta C_T}$ threshold cycle (C_T) method. Means ($n = 4$ independent RNA samples for *prtS* and 7 for *slpB*) and standard deviations are shown; asterisks indicate statistically significant differences from WT results determined by the Mann-Whitney U-test ($P = 0.0286$ for *prtS* and $P = 0.0012$ for *slpB*). (B) EMSA analysis of MBP-EepR interaction with the biotin-labeled *prtS* promoter (PprtS) (2 ng) *in vitro*. Poly(dI-dC) (500 ng) was included to prevent nonspecific binding. Unlike MBP-EepR, recombinant MBP was unable to produce a gel shift, indicating that the EepR portion of the fusion is necessary for binding to PprtS. The experiment was performed three times with similar pattern results. PprtS-UL indicates the same promoter region but without a biotin label. An excess of unlabeled *prtS* promoter outcompeted the biotin-bound promoter, suggesting specificity for the *prtS* promoter.

sus A and B) (22). Mutation of *eepR* in strain K904 reduced secreted-protease activity by approximately 50%, and this defect could be complemented by the presence of an intact *eepR* gene on a plasmid, suggesting that the defect was due to loss of *eepR* and not due to a polar effect or another mutation (Fig. 1C). An *eepR* deletion mutation in a third strain, Nima (28), also conferred a protease defect on milk agar plates (clearing radius of 7.8 ± 0.6 mm for the Nima strain versus no zone for the Nima $\Delta eepR$ strain; $n = 8$), further suggesting a conserved role for EepR in promoting protease production.

Positive transcriptional regulators of *prtS* expression have not been reported. To test whether *prtS* transcription is activated by EepR, qPCR was used with RNA extracted from the $\Delta eepR$ strain (CMS2097) versus the WT (CMS376) cultures grown to an OD₆₀₀ of 3. Expression of the *prtS* gene was down 5-fold in the *eepR* mutant compared to the WT (Fig. 2A). EMSA analysis provided evidence that recombinant EepR binds to DNA directly upstream of *prtS* (see Fig. S2B in the supplemental material). In addition to the use of poly(dI-dC) to prevent nonspecific interactions, a previously published study performed with identical reagents showed that MBP-EepR does not bind to the *gdhS* promoter or the *pigP* promoter, supporting the model that EepR specifically binds to the *prtS* promoter (26).

A recent study identified another cytotoxic metalloprotease secreted by *S. marcescens*, SlpB (22). SlpB was highly cytotoxic to an airway cell line but only minimally toxic to a corneal cell line. Similarly to what was observed with *prtS*, expression of *slpB* was highly reduced in the $\Delta eepR$ mutant compared to the WT

FIG 3 Reduced levels of metalloprotease and surface layer protein secreted by the $\Delta eepR$ mutant. A representative Coomassie-stained PAGE gel of secreted fractions of strain K904 and isogenic mutant strains is shown. The top arrow indicates SlaA protein, and the bottom arrow indicates both the PrtS and SlpB proteins.

CMS376 strain (Fig. 2C). The level of *slpB* transcript was more than 10-fold lower in a K904 $\Delta eepR$ mutant than in strain K904 ($P < 0.05$ [Mann-Whitney]).

When secreted proteins of strain K904 were separated on a PAGE gel (Fig. 3), there was a clear band at ~50 kDa that was highly reduced in the K904 $\Delta prtS$ mutant and restored when *prtS* was induced from a plasmid in the $\Delta prtS$ strain (Fig. 3; see also Fig. S1A in the supplemental material). The ~50-kDa band was reduced in the $\Delta eepR$ mutant and restored when *eepR* or *prtS* was expressed from a plasmid (Fig. 3; see also Fig. S1A). As reported previously (22), using another portion of the gel shown in Fig. 3, mass spectrometry identified the ~50-kDa bands from the $\Delta prtS$ and $\Delta slpB$ strains as SlpB and PrtS, respectively (Fig. 3). Deletion of both *prtS* and *slpB* abolished the ~50-kDa band (data not shown) (22). Together, these genetic and biochemical data suggest that EepR positively regulates production of secreted metalloproteases.

Mutation of *eepR* suppresses the hyperprotease phenotype of a *crp* mutant. Previous reports showed that the broadly conserved cyclic-AMP receptor protein (CRP) regulates pigmentation and hemolysis in an EepR-dependent manner and that mutations in the *crp* gene yield cells with elevated levels of secreted proteolysis (17, 26) (Fig. 1A). Here, we used double mutants to genetically assess whether CRP and EepR share a pathway of regulating the secreted protease(s). The *crp eepR* (CMS2157) double-mutant strain exhibited reduced secreted-protease activity compared to the *crp* mutant (Fig. 1A), suggesting that CRP and EepR act in a common pathway to regulate protease production.

Secreted chitinase activity is mediated by EepR. The aforementioned PAGE gel had another band (~100 kDa) that suggested reduced expression in the $\Delta eepR$ strain and that was restored by expression of *eepR* *in trans* (Fig. 3; see also Fig. S1B in the supplemental material). This protein was identified by mass spectrometry as the secreted S-layer protein SlaA. The altered expression of proteases and SlaA impelled us to test whether EepR had a more global impact on production of secreted proteins. Two-dimensional difference gel electrophoresis (2D-DIGE) analysis was performed on secreted proteins from the WT strain (CMS376) and the $\Delta eepR$ mutant grown to saturation in LB medium (see Fig. S2). A subset of proteins, with staining intensities observed by comparisons of 2D-DIGE results from the two strains, were iden-

FIG 4 Secreted chitinase activity and *cbp21* expression are modulated by EepR. (A) Expression of *cbp21* was significantly reduced in the *ΔeepR* mutant compared to the wild type in bacterial cells grown to an OD₆₀₀ of 3 in LB medium. Means and standard deviations of the results of 4 separate experiments are shown. The values are relative to 16S expression and were quantified using the $2^{-\Delta\Delta CT}$ method. Asterisks indicate statistically significant differences from WT results determined by the Mann-Whitney U-test ($P = 0.0159$). (B) Representative image of complementation of the *ΔeepR* mutant defect on chitin azure agar plates. Vector, pMQ131; *eepR*, pMQ364. The arrowhead indicates the zone of chitinase activity. (C) Secreted chitinase activity measured from spent culture supernatants using chitinase azure. Averages and standard deviations are shown ($n \geq 6$). LB medium was used as a negative control. Asterisks indicate statistically significant differences from the LB results determined by ANOVA with Tukey's posttest ($P < 0.001$). WT, CMS376; *eepR*, CMS2097; K904, CMS853; K904 *eepR*, CMS2904.

tified by mass spectrometry. Among the proteins with decreased expression in the *ΔeepR* mutant was the serralsin protease, PrtS (expression reduced 2.9-fold in the *ΔeepR* strain) (see Fig. S2), supporting the observations presented in Fig. 3 (see also Fig. S1A). Other proteins with differential production results included chitinase A, chitinase B, chitinase C, and chitin binding protein 21 (Cbp21) (see Fig. S2). All had a greater than 5-fold reduction in expression (see Fig. S2). Similarly to the decrease in protein levels, the transcript of a chitin-binding protein gene, *cbp21*, was measured from cultures at an OD₆₀₀ of 3.0 using qPCR. A 133-fold reduction in the level of the *eepR* mutant compared to the level of the WT (CMS376) was measured (Fig. 4A), supporting the findings of the 2D-DIGE analysis.

Consistent with the observed reduction in the chitin-processing proteins, the *ΔeepR* mutant had reduced levels of secreted chitinase activity with no zone of clearance evident on chitin azure agar plates (Fig. 4B). This activity could be complemented by the *eepR* wild-type gene expressed in *trans* in both the K904 and CMS376 backgrounds (Fig. 4B and data not shown). A 3-fold-to-

FIG 5 EepR is necessary for cytotoxicity of *S. marcescens* against HCLE cells. (A) Cytotoxicity of bacteria (MOI = 10) from the WT strain (CMS376) and clinical isolate K904 (CMS853) and isogenic *eepR* mutants against HCLE cells. (B) Data are presented as described for panel A but represent the cytotoxicity of bacterial supernatants to HCLE cells ($n \geq 8$). Asterisks indicate statistically significant differences between the K904 and the *eepR* mutant results determined by ANOVA with Tukey's posttest ($P < 0.001$). (C) The cytotoxicity of normalized filtered bacterial supernatants to HCLE cells was measured using alamarBlue ($n = 6$). vector, pMQ132; *eepR*, pMQ369. Means and standard deviations are shown. Asterisks indicate statistically significant differences from K904 plus vector determined by ANOVA with Tukey's posttest ($P < 0.001$). (D) Cytotoxicity of K904 supernatants under either normal conditions or heat treatment conditions (95°C for 10 min) ($n \geq 7$). Asterisks indicate statistically significant differences between groups determined using a two-tailed Student's *t* test ($P < 0.0001$).

4-fold reduction in secreted chitinase activity was measured from the *eepR* deletion mutant relative to the WT strain (CMS376) ($P < 0.001$ [ANOVA with Tukey's posttest]), and a similar trend was observed with the K904 and K904 *ΔeepR* strains (Fig. 4C). Together, these results indicated that EepR is an important regulator of secreted proteins.

Mutation of *eepR* in clinical isolate K904 reduces the cytotoxicity of bacterial secretomes for human epithelial cells *in vitro*.

As a primary assessment of the role of EepR in bacterial virulence, the WT strain (CMS376) and the *eepR* mutant were tested for cytotoxicity in monolayers of the human ocular HCLE cell line. *S. marcescens* can be cytotoxic to eukaryotic cells via the activity of a surface-attached cytotoxin and through that of secreted enzymes; therefore, we tested both bacterial cells (at an MOI of 10) and secreted supernatants for cytotoxic potential. The WT (CMS376) and *eepR* mutant strains and normalized filter-sterilized spent supernatant were used to challenge HCLE cells for 2 h (bacterial cells) or 4 h (supernatants), and then HCLE cell viability was assessed using a fluorescent viability dye (Fig. 5A). In this and the following cytotoxicity experiments, a mock control with no bacteria had 0% cytotoxicity (data not shown). Unlike strain

FIG 6 Induced serralyisin production restores cytotoxic capacity to *eepR* mutant supernatants. (A) Protease activity from normalized bacterial supernatants measured using azocasein ($n = 3$). Averages and standard deviations are shown. (B) Data are presented as described for panel A, but cytotoxicity to HCLE cells was measured ($n = 3$). For both charts, asterisks indicate a statistically significant difference from the results for K904 plus vector determined by ANOVA with Tukey's posttest ($P < 0.001$). K904, CMS853; K904 *eepR*, CMS2904; vector, pMQ125; *prtS*, pMQ356.

CMS376, bacteria of the K904 clinical isolate and an isogenic *eepR* deletion strain were highly and similarly cytotoxic (Fig. 5A). This difference in cytotoxicity between isolates was previously shown (40) and correlated with differences in secreted-protease production.

Bacterial supernatants derived from strain CMS376 and those from the $\Delta eepR$ mutant produced the same pattern: minimal cytotoxicity (Fig. 5B). Strikingly, whereas the K904 supernatant was highly cytotoxic to HCLE cells, the K904 *eepR* supernatant produced little measurable cytotoxicity (Fig. 5B). Expression of the *eepR* gene on a plasmid could restore cytotoxicity to supernatants from the K904 $\Delta eepR$ strain (Fig. 5C).

The cytotoxic secreted supernatant from strain K904 was heat treated (95°C for 10 min) to gain insight into its nature. The heat-treated secreted supernatant was highly reduced in cytotoxicity to HCLE cells (Fig. 5D). This suggested that a heat-labile factor, such as a protein, was required for cytotoxicity. Given that serralyisin was previously reported to be cytotoxic to HCLE cells (22) and that its expression is reduced in the *eepR* mutant, we tested the prediction that the K904 *eepR* supernatants are less cytotoxic because of reduced serralyisin levels due to decreased *prtS* expression. Figure 6 illustrates that induced expression of *prtS* could restore secreted proteolysis to nearly wild-type levels and could complement the cytotoxicity defect of K904 $\Delta eepR$ mutant supernatants. These data indicate that EepR is required for a *S. marcescens* clinical isolate to secrete cytotoxic factors and support the idea that expression of *prtS* is sufficient to restore cytotoxicity.

EepR mediates *S. marcescens* induction of proinflammatory cytokine IL-1 β expression. *S. marcescens* induces a strong inflammatory response in ocular models (41, 42). Purified serralyisin induces cytokine expression *in vitro* (43). We therefore determined whether mutation of *eepR* would influence the proinflammatory capacity of *S. marcescens* culture supernatants using a commercial cytokine array. After coincubation of the bacterial supernatants with HCLE cells, proinflammatory markers were assessed using a commercial cytokine array (see Fig. S3 in the supplemental material). Compared to the LB (mock) control used to determine baseline expression of proinflammatory markers, the WT (CMS376) strain-exposed HCLE cells produced an increase in the levels of IL-1 α , IL-1 β , IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas the results ob-

FIG 7 EepR is required to elicit full levels of IL-1 β production from HCLE cells. IL-1 β levels were measured in HCLE cells exposed to normalized bacterial supernatants for 20 h. Averages and standard deviations are shown ($n \geq 6$). Asterisks indicate statistically significant differences from mock infection (LB medium addition) results determined by ANOVA with Tukey's posttest ($P < 0.001$).

served with the $\Delta eepR$ mutant supernatant were similar to those seen under mock conditions (see Fig. S3A and B).

IL-1 β is a key mediator of the host response to ocular infections and other infections (42, 44, 45). Therefore, we used an ELISA to verify the cytokine array data for this important proinflammatory cytokine. Levels of IL-1 β released from HCLE cells were significantly reduced ($P < 0.05$) after exposure to *eepR* mutant (CMS2097) supernatants compared to those observed after exposure to the WT strain (CMS376) (Fig. 7). However, this effect was not observed with the K904 strain background, where similar levels of IL-1 β were measured in cells exposed to K904 and the K904 $\Delta eepR$ mutant supernatants (data not shown), suggesting a strain-dependent phenotype.

EepR is not required for keratitis but is necessary for proliferation in a corneal-infection rabbit model. The K904 strain and the isogenic K904 $\Delta eepR$ mutant were tested for virulence in a rabbit model of keratitis (46, 47). K904 was chosen over CMS376 because it was derived from a human keratitis patient. Bacteria (~100 CFU) were injected into the corneal stroma of New Zealand White rabbits *in vivo*. At 48 h after injection, eyes were evaluated for clinical scores by masked observation as previously described (48, 49), and bacterial counts in the corneas were determined. By 48 h, clinical scores were highly similar for the eyes infected with K904 and those infected with the K904 $\Delta eepR$ mutant ($P > 0.05$) and significantly different from those determined for the mock-treated eyes (Fig. 8A) ($P < 0.05$ [Kruskal-Wallis test with Dunn's multiple-comparison test]). Injection of PBS (Mock) resulted in a median clinical score of 0 (with individual scores ranging from 0 to 4), injection of the K904 strain resulted in a median clinical score of 15 with a range from 13 to 18, and injection of the K904 $\Delta eepR$ mutant resulted in a median clinical score of 15.75 with a range from 12 to 18 ($n = 8$ rabbits and eyes per condition). The bacteria were highly inflammatory, and the clinical signs included large corneal ulcers, hypopyons in the anterior chamber, corneal edema, iritis, and chemosis of the conjunctiva. However, bacterial counts (Fig. 8B) were 10-fold lower from corneas infected with the $\Delta eepR$ mutant ($6.63 \pm 0.28 \log_{10}$ CFU) than from those infected with the K904 strain ($7.68 \pm 0.37 \log_{10}$ CFU; $n = 6$ rabbits per strain from two separate experiments performed with one eye per rabbit) ($P = 0.046$ [Student's *t* test]).

Rabbit corneas, infected exactly as described above but with strain K904 and with K904 with an *eepR* insertion mutation rather

FIG 8 Evaluation of an *eepR* mutant in a rabbit keratitis model. (A) Combined clinical signs of inflammation, including chemosis, iritis, and corneal ulcer, 48 h following injection of ~ 100 bacteria into each cornea ($n = 8$ rabbits). Median and range values are shown. (B) CFU determination of bacteria from corneas of rabbits sacrificed 48 h after intrastromal injection ($n = 6$). Means and standard deviations are shown. The asterisk indicates a statistically significant difference determined by Student's *t* test ($P = 0.0459$).

than the deletion mutation, also failed to achieve wild-type levels of CFU *in vivo*. At 48 h after injection into the rabbit cornea, strain K904 achieved 7.48 ± 0.05 ($\log_{10}$) CFU compared to 6.91 ± 0.04 ($\log_{10}$) CFU for the K904 *eepR* insertion mutant ($n = 2$ rabbits and eyes per strain) ($P = 0.01$ [Student's *t* test]). Strain CMS2862, in which the $\Delta eepR$ deletion allele of CMS2904 had been replaced with the wild-type *eepR* allele, was used to control for the possibility that mutations elsewhere on the chromosome were responsible for the *eepR* mutant corneal-proliferation defect. When 3 rabbit corneas were injected with 100 CFU of strain CMS2862, the CFU enumerated from the rabbit eyes at 48 h were similar to what was observed with the wild-type K904 strain, 8.00 ± 0.18 ($\log_{10}$) CFU, supporting the idea that the *eepR* mutation was responsible for the proliferation defect.

Importantly, a previous study showed that the K904 WT strain and the same K904 $\Delta eepR$ mutant strain grew at the same rates in minimal and rich media, indicating that the reduction in proliferation was not due to a slow-growth phenotype (26). These results suggest that EepR is required to achieve wild-type levels of proliferation during an *in vivo* corneal infection.

Desiccation survival, but not heat tolerance or hydrogen peroxide resistance, is decreased in an *eepR* mutant. The ability of bacteria to survive desiccation is important for the success of nosocomial opportunistic pathogens (50, 51). *S. marcescens* desiccation survival was tested using the wild-type (CMS376) strain and the $\Delta eepR$ mutant (CMS2097) strain. Bacteria grown in LB medium were normalized by optical density; aliquots were applied in a thin film in multiwell plates and allowed to dry in a laminar flow hood for 20 min and then placed at 30°C. After 24 h, fresh medium was added and pipetted up and down several times, and the CFU of the surviving bacteria were determined after serial dilution plating (Fig. 9A). There was a more than 2 $\log_{10}$ reduction in the frequency of viable *eepR* mutant cells recovered in this assay (Fig. 9A).

This result suggests that the $\Delta eepR$ mutant is much less able to survive desiccation stress than the wild type; however, it could be that the $\Delta eepR$ mutant remains attached to the plastic surface better than the wild type, resulting in an underestimation of the actual number of mutant bacteria involved. To differentiate between these two scenarios, 10-fold serial dilutions of normalized wild-type and $\Delta eepR$ mutant cultures were added to multiple wells

FIG 9 EepR is necessary for desiccation stress survival but not heat stress survival. (A) Frequency of surviving bacteria after 24 h of desiccation at 30°C compared to the inoculum. The $\Delta eepR$ mutant survival frequency was significantly different from that of the WT strain determined by Student's *t* test, as indicated by the asterisk ($P = 0.0332$; $n \geq 4$). (B) Cells were serially diluted, desiccated, and incubated for 24 h at 30°C. LB medium was then added to wells, and turbidity was measured after 48 h at 30°C ($n = 3$). (C) Bacterial survival after treatment at 65°C. For each chart, the means and standard deviations are shown ($n = 6$).

in a microtiter plate. After desiccation challenge, 100 μ l of LB medium was added to each well, and the plate was incubated to allow growth of the surviving cells. After 48 h of incubation at 30°C, the turbidity of the cultures was measured spectrophotometrically. For the $\Delta eepR$ mutant, growth was evident in wells down to a dilution of 10⁻³, whereas growth was evident in wells down to a dilution of 10⁻⁴ for the wild type (Fig. 8B). Similarly to what was observed in the CMS376 background, the *eepR* gene was found to be necessary for full desiccation survival in the K904 background. Normalized cultures of the K904 strain, the K904 $\Delta eepR$ mutant, and the $\Delta eepR$ mutant with the restored *eepR* gene (CMS2862) were dried for 24 h followed by 24 h of growth in LB medium. Culture density (OD₆₀₀) was measured at 0.44 ± 0.02 for K904, 0.12 ± 0.05 for the K904 $\Delta eepR$ mutant, and 0.31 ± 0.03 for CMS2862. The K904 $\Delta eepR$ mutant had significantly less growth after desiccation challenge than the K904 and CMS2862 strains ($P < 0.01$ [ANOVA with Tukey's posttest]). The dilution desiccation experiment followed similar patterns with CMS376 (Fig. 9B) and K904 (see Fig. S4A in the supplemental material). Together, these experiments demonstrated a role for EepR in desiccation stress survival.

Because CRP and EepR appear to be in a common pathway for regulation of secreted-protease activity (Fig. 1) and of secondary metabolites serratamolide and prodigiosin (26), we tested whether CRP had a role in desiccation survival using a *crp* deletion mutant strain isogenic to CMS376 (CMS1687) (see Fig. S4B in the supplemental material). The optical density achieved by desiccated cultures (24 h) was significantly higher ($P < 0.01$) for the *crp* mutant (CMS1687) than for the wild type (CMS376) (OD₆₀₀ = 0.34 ± 0.06 for the *crp* mutant and 0.20 ± 0.07 for the wild type). A *crp eepR* double mutant (CMS2157) survived at a

level that was similar to that seen with the wild type ($P > 0.05$) and significantly lower than that seen with the *crp* mutant ($P < 0.01$) ($OD_{600} = 0.19 \pm 0.01$). A strain in which the $\Delta eepR$ allele was replaced by the wild-type *eepR* gene (CMS2921) was able to grow to a level comparable to that seen with the isogenic wild type (CMS376) ($OD_{600} = 0.17 \pm 0.06$).

The *eepR* mutant was previously shown to be defective in production of both prodigiosin and serratamolide, which are lipid-associated secondary metabolites that could have an impact upon desiccation survival (26). We tested the prediction that prodigiosin and serratamolide were necessary for desiccation survival using strains with mutations in *pigA* and *swrW*, which are necessary for biosynthesis of the respective secondary metabolites (26). The desiccation defect does not appear to be due to loss of prodigiosin pigment or serratamolide surfactant from the $\Delta eepR$ mutant, as a *pigA* mutant strain and a *swrW* mutant strain had survival profiles similar to those seen with mutant strains of the isogenic parental K904 strain (OD_{600} of 0.57 ± 0.13 for K904, 0.61 ± 0.12 for the *swrW* mutant, and 0.63 ± 0.11 for the *pigA* mutant [$n = 3$]).

To test whether EepR has a role in enduring other stresses, 10^8 CFU of the wild-type strain and 10^8 CFU of the $\Delta eepR$ strain were challenged by incubation at high temperature (65°C). The losses of viability were indistinguishable for the two strains. At the 20-min time point, less than 10^4 CFU remained viable, and by 1 h, the levels of viable cells had fallen below the limit of detection (100 CFU/ml) for both strains (Fig. 8C). Similarly to the heat tolerance results, the $\Delta eepR$ mutant was identical to the wild type with respect to hydrogen peroxide susceptibility. The average zones of clearance (in millimeters) around paper discs imbued with 30% hydrogen peroxide on LB agar plates were 22.3 ± 1.2 for the WT strain (CMS376) and 22.4 ± 0.6 for the $\Delta eepR$ mutant ($n = 6$ for each).

DISCUSSION

This report provides the first example of a positive transcriptional regulator of serralyisin. Biochemical and transcriptional analysis data suggest that EepR regulates a number of chitin-metabolizing enzymes, Cbp21, metalloproteases PrtS and SlpB, and surface layer protein SlaA, suggesting that EepR is a global regulator of secreted proteins. Beyond serralyisin, these secreted proteins may play a role in virulence. Recent studies have indicated a role for bacterial chitinases and chitin binding proteins in host-pathogen interactions and virulence as reviewed by Frederiksen et al. (52). For example, chitinase B from *S. marcescens* can hydrolyze human mucins which may provide better access to epithelial cell surfaces (53). Consistent with this idea, *chiA* mutants of other species of *Enterobacteriaceae* are less able to attach to epithelial cells *in vitro* (52). The N terminus of GbpA from *Vibrio cholerae* shares ~50% amino acid sequence identity with Cbp21 of *S. marcescens*; mutation of the genes for these polysaccharide binding proteins conferred a reduction in attachment to epithelial cells *in vitro* (54, 55). Therefore, it is possible that the chitinases and chitin binding proteins controlled by EepR contribute to pathogenesis under certain circumstances. This may be especially important in insect pathogenesis. In agreement with this prediction, several researchers have employed expression of a variety of *S. marcescens* chitinase genes, *chiA*, *chiB*, and *chiC*, to enhance the insecticidal activity of *Bacillus thuringiensis* and other bacteria (56–58).

The outcome of this study and that of a previous one that demonstrated direct transcriptional control of *eepR* expression by

cAMP-CRP (26) link a highly conserved metabolic regulator, the catabolite repression system, to regulation of factors that likely aid in nutrient acquisition and competition. More specifically, the metalloproteases and chitinases may make nutrients available by breaking down polymers into molecules more easily transported into the bacterium. Since cAMP levels are reduced under high-nutrient conditions, it is expected that these EepR levels would increase, leading to greater production of these nutrient acquisition factors. CRP is also a major virulence factor in a number of organisms, and the cAMP-EepR pathway may be of importance in the virulence of other organisms with EepR-like proteins such as AtsT from *Burkholderia* species (59). Furthermore, our observations suggest that CRP and EepR have opposing roles in regulating desiccation stress survival through a mechanism that is unknown at this time.

It is of note that the *in vitro* inflammation studies demonstrated levels of IL-1 β and other inflammatory markers following challenge of corneal cells with $\Delta eepR$ mutant supernatants that were lower than those from the WT in the CMS376 strain background, but the pattern was not followed with the K904 strain. These outcomes may be explained based on strain differences, as the K904 strain generates approximately 20 times more secreted-protease activity than strain CMS376 (Fig. 1) and because *S. marcescens* secreted proteases are highly inflammatory (43). The K904 $\Delta eepR$ mutant, while reduced in protease secretion compared to the K904 strain, still produces ~10 times more protease than CMS376. The K904 $\Delta eepR$ mutant is likely to be more inflammatory than the CMS376 wild-type strain, as determined on the basis of protease production alone; it may also produce other inflammatory products not made by strain CMS376.

The K904 and K904 $\Delta eepR$ strains were both highly inflammatory in the rabbit cornea based on clinical signs, despite the difference in levels of proliferation in the rabbit cornea. Given that the K904 $\Delta eepR$ mutant achieved >1 million CFU/cornea, that it still generates levels of proinflammatory proteases that are ~50% of wild-type levels, and that inflammation is the major cause of pathology in bacterial keratitis (42, 44, 45), it was not particularly surprising that the K904 $\Delta eepR$ mutant caused clinical outcomes similar to those observed with the parental strain. The infection model used here maximized the reproducibility of the inoculum by injecting the bacteria into the cornea and is good for determining the survival and proliferation of bacteria in the cornea; however, it may underestimate the differences between strains where virulence factors involved in attachment, invasion, and inflammation are important.

Whereas secreted-protease production and desiccation survival were highly dependent on the presence of EepR in both tested strains, the differences between strains demonstrated here underscore an emerging theme, that using only one strain of a bacterial species to test the role of a gene in a process is less than ideal (39). The two strains used here were chosen because of their differences. For example, K904 was the strain with highest levels of secreted-protease production and cytotoxicity among ~50 tested *S. marcescens* strains in a recent study, whereas CMS376 was among those with the lowest levels (22). These differences facilitate a more rigorous assessment of the role of EepR in virulence-related phenotypes and provide insight into the complexity and diversity of regulation of virulence by *S. marcescens*. Results from this study indicate that EepR was important for protease and chitinase production in both CMS376 and K904 but was required for inducing IL-1 β only in CMS376. In a previous study, EepR was shown to be important for production of the secondary metabo-

lites prodigiosin and serratamolide in strains CMS376, K904, and Nima (26). Together, these results support the idea of a conserved role for EepR among diverse *S. marcescens* strains, with the exception of IL-1 β stimulation.

Two-component regulator proteins have been broadly implicated in pathogenesis (60–62), but this has been less well studied with ocular pathogenesis. Until this study, the only example of a two-component regulator being involved in ocular pathogenesis was provided by the Fleiszig group using a *retS* transposon mutant strain of *Pseudomonas aeruginosa* (62). RetS, a hybrid sensor-kinase involved in type III secretion system regulation, was necessary for full levels of virulence in a mouse model of infection and cytotoxicity in rabbit corneal cells *in vitro* (62). The present report supports the idea that EepR also has a role in ocular virulence and suggests a common role for hybrid two-component regulators in regulation of ocular virulence.

In summary, the results from this study support the idea that EepR is a positive regulator of secreted cytotoxic metalloproteases and chitinases. EepR is necessary for survival of medically relevant desiccation stress survival and is involved in proliferation in corneal tissue. Therefore, linked to a previous study connecting EepR to the highly conserved cAMP-CRP pathway and indicating its importance in regulation of the secreted cytolytic surfactant serratamolide and the antimicrobial pigment prodigiosin, this report implicates EepR as a global regulator of secreted enzymes and secondary metabolites.

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REFERENCES

- Butterworth MB, Zhang L, Liu X, Shanks RM, Thibodeau PH. 2014. Modulation of the epithelial sodium channel (ENaC) by bacterial metalloproteases and protease inhibitors. *PLoS One* 9:e100313. <http://dx.doi.org/10.1371/journal.pone.0100313>.
- Limoli DH, Sladek JA, Fuller LA, Singh AK, King SJ. 2011. BgaA acts as an adhesin to mediate attachment of some pneumococcal strains to human epithelial cells. *Microbiology* 157:2369–2381. <http://dx.doi.org/10.1099/mic.0.045609-0>.
- Ruiz-Perez F, Nataro JP. 2014. Bacterial serine proteases secreted by the autotransporter pathway: classification, specificity, and role in virulence. *Cell Mol Life Sci* 71:745–770. <http://dx.doi.org/10.1007/s00018-013-1355-8>.
- Schmiel DH, Miller VL. 1999. Bacterial phospholipases and pathogenesis. *Microbes Infect* 1:1103–1112. [http://dx.doi.org/10.1016/S1286-4579\(99\)00205-1](http://dx.doi.org/10.1016/S1286-4579(99)00205-1).
- Zdzalik M, Karim AY, Wolski K, Buda P, Wojcik K, Brueggemann S, Wojciechowski P, Eick S, Calander AM, Jonsson IM, Kubica M, Polakowska K, Miedzobrodzki J, Wladyka B, Potempa J, Dubin G. 2012. Prevalence of genes encoding extracellular proteases in *Staphylococcus aureus*—important targets triggering immune response *in vivo*. *FEMS Immunol Med Microbiol* 66:220–229. <http://dx.doi.org/10.1111/j.1574-695X.2012.01005.x>.
- Richards MJ, Edwards JR, Culver DH, Gaynes RP. 2000. Nosocomial infections in combined medical-surgical intensive care units in the United States. *Infect Control Hosp Epidemiol* 21:510–515. <http://dx.doi.org/10.1086/501795>.
- Samonis G, Vouloumanou EK, Christofaki M, Dimopoulou D, Maraki S, Triantafyllou E, Kofteridis DP, Falagas ME. 2011. *Serratia* infections in a general hospital: characteristics and outcomes. *Eur J Clin Microbiol Infect Dis* 30:653–660. <http://dx.doi.org/10.1007/s10096-010-1135-4>.
- Mahlen SD. 2011. *Serratia* infections: from military experiments to current practice. *Clin Microbiol Rev* 24:755–791. <http://dx.doi.org/10.1128/CMR.00017-11>.
- Merkier AK, Rodriguez MC, Togneri A, Brengi S, Osuna C, Pichel M, Cassini MH, Centron D. 2013. Outbreak of a cluster with epidemic behavior due to *Serratia marcescens* after colistin administration in a hospital setting. *J Clin Microbiol* 51:2295–2302. <http://dx.doi.org/10.1128/JCM.03280-12>.
- Bertrand X, Dowzicky MJ. 2012. Antimicrobial susceptibility among gram-negative isolates collected from intensive care units in North America, Europe, the Asia-Pacific Rim, Latin America, the Middle East, and Africa between 2004 and 2009 as part of the Tigecycline Evaluation and Surveillance Trial. *Clin Ther* 34:124–137. <http://dx.doi.org/10.1016/j.clinthera.2011.11.023>.
- Das S, Sheorey H, Taylor HR, Vajpayee RB. 2007. Association between cultures of contact lens and corneal scraping in contact lens related microbial keratitis. *Arch Ophthalmol* 125:1182–1185. <http://dx.doi.org/10.1001/archophth.125.9.1182>.
- Mah-Sadorra JH, Najjar DM, Rapuano CJ, Laibson PR, Cohen EJ. 2005. *Serratia* corneal ulcers: a retrospective clinical study. *Cornea* 24:793–800. <http://dx.doi.org/10.1097/01.icc.0000159738.06167.88>.
- Hume EB, Willcox MD. 2004. Emergence of *Serratia marcescens* as an ocular surface pathogen. *Arch Soc Exp Ophthalmol* 79:475–477.
- Watanabe T, Kimura K, Sumiya T, Nikaidou N, Suzuki K, Suzuki M, Taiyaji M, Ferrer S, Regue M. 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *J Bacteriol* 179:7111–7117.
- Jin S, Chen Y, Christie GE, Benedik MJ. 1996. Regulation of the *Serratia marcescens* extracellular nuclease: positive control by a homolog of P2 Ogr encoded by a cryptic prophage. *J Mol Biol* 256:264–278. <http://dx.doi.org/10.1006/jmbi.1996.0084>.
- Marty KB, Williams CL, Guynn LJ, Benedik MJ, Blanke SR. 2002. Characterization of a cytotoxic factor in culture filtrates of *Serratia marcescens*. *Infect Immun* 70:1121–1128. <http://dx.doi.org/10.1128/IAI.70.3.1121-1128.2002>.
- Shanks RM, Stella NA, Arena KE, Fender JE. 2013. Mutation of *crp* mediates *Serratia marcescens* serralyisin and global secreted protein production. *Res Microbiol* 164:38–45. <http://dx.doi.org/10.1016/j.resmic.2012.10.006>.
- Kalivoda EJ, Stella NA, Aston MA, Fender JE, Thompson PP, Kowalski RP, Shanks RM. 2010. Cyclic AMP negatively regulates prodigiosin production by *Serratia marcescens*. *Res Microbiol* 161:158–167. <http://dx.doi.org/10.1016/j.resmic.2009.12.004>.
- Shanks RM, Stella NA, Lahr RM, Wang S, Veverka TI, Kowalski RP, Liu X. 2012. Serratamolide is a hemolytic factor produced by *Serratia marcescens*. *PLoS One* 7:e36398. <http://dx.doi.org/10.1371/journal.pone.0036398>.
- Ishii K, Adachi T, Hara T, Hamamoto H, Sekimizu K. 2014. Identification of a *Serratia marcescens* virulence factor that promotes hemolymph bleeding in the silkworm, *Bombyx mori*. *J Invertebr Pathol* 117:61–67. <http://dx.doi.org/10.1016/j.jip.2014.02.001>.
- Molla A, Matsumoto K, Oyama I, Katsuki T, Maeda H. 1986. Degradation of protease inhibitors, immunoglobulins, and other serum proteins by *Serratia* protease and its toxicity to fibroblast in culture. *Infect Immun* 53:522–529.
- Shanks RM, Stella NA, Hunt KM, Brothers KM, Zhang L, Thibodeau PH. 2015. Identification of SlpB, a cytotoxic protease from *Serratia marcescens*. *Infect Immun* 83:2907–2916. <http://dx.doi.org/10.1128/IAI.03096-14>.
- Matsumoto K. 2004. Role of bacterial proteases in pseudomonal and serratal keratitis. *Biol Chem* 385:1007–1016.
- Ishii K, Adachi T, Hamamoto H, Sekimizu K. 2014. *Serratia marcescens* suppresses host cellular immunity via the production of an adhesion-inhibitory factor against immunosurveillance cells. *J Biol Chem* 289:5876–5888. <http://dx.doi.org/10.1074/jbc.M113.544536>.
- Akaike T, Molla A, Ando M, Araki S, Maeda H. 1989. Molecular mechanism of complex infection by bacteria and virus analyzed by a model using serratal protease and influenza virus in mice. *J Virol* 63:2252–2259.
- Stella NA, Lahr RM, Brothers KM, Kalivoda EJ, Hunt KM, Kwak DH, Liu

- X, Shanks RM. 2015. *Serratia marcescens* cyclic AMP-receptor protein controls transcription of EepR, a novel regulator of antimicrobial secondary metabolites. *J Bacteriol* 197:2468–2478. <http://dx.doi.org/10.1128/JB.00136-15>.
27. Kalivoda EJ, Stella NA, O'Dee DM, Nau GJ, Shanks RM. 2008. The cyclic AMP-dependent catabolite repression system of *Serratia marcescens* mediates biofilm formation through regulation of type 1 fimbriae. *Appl Environ Microbiol* 74:3461–3470. <http://dx.doi.org/10.1128/AEM.02733-07>.
28. Green JA, Rappoport DA, Williams RP. 1956. Studies on pigmentation of *Serratia marcescens*. II. Characterization of the blue and the combined red pigments of prodigiosin. *J Bacteriol* 72:483–487.
29. Shanks RM, Lahr RM, Stella NA, Arena KE, Brothers KM, Kwak DH, Liu X, Kalivoda EJ. 2013. A *Serratia marcescens* PigP homolog controls prodigiosin biosynthesis, swarming motility and hemolysis and is regulated by cAMP-CRP and HexS. *PLoS One* 8:e57634. <http://dx.doi.org/10.1371/journal.pone.0057634>.
30. Shanks RM, Kadouri DE, MacEachran DP, O'Toole GA. 2009. New yeast recombineering tools for bacteria. *Plasmid* 62:88–97. <http://dx.doi.org/10.1016/j.plasmid.2009.05.002>.
31. Kalivoda EJ, Horzempa J, Stella NA, Sadaf A, Kowalski RP, Nau GJ, Shanks RM. 2011. New vector tools with a hygromycin resistance marker for use with opportunistic pathogens. *Mol Biotechnol* 48:7–14. <http://dx.doi.org/10.1007/s12033-010-9342-x>.
32. Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 62:293–300.
33. Miller VL, Mekalanos JJ. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* 170:2575.
34. Cruz-Romero M, Kelly AL, Kerry JP. 2008. Influence of packaging strategy on microbiological and biochemical changes in high-pressure-treated oysters. *J Sci Food Agric* 88:2713–2723. <http://dx.doi.org/10.1002/jsfa.3398>.
35. Shanks RM, Stella NA, Kalivoda EJ, Doe MR, O'Dee DM, Lathrop KL, Guo FL, Nau GJ. 2007. A *Serratia marcescens* OxyR homolog mediates surface attachment and biofilm formation. *J Bacteriol* 189:7262–7272. <http://dx.doi.org/10.1128/JB.00859-07>.
36. Tshala-Katumbay D, Monterroso V, Kayton R, Lasarev M, Sabri M, Spencer P. 2008. Probing mechanisms of axonopathy. Part I. Protein targets of 1,2-diacetylbenzene, the neurotoxic metabolite of aromatic solvent 1,2-diethylbenzene. *Toxicol Sci* 105:134–141.
37. Gipson IK, Spurr-Michaud S, Argüeso P, Tisdale A, Ng TF, Russo CL. 2003. Mucin gene expression in immortalized human conreal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci* 44:2496–2506. <http://dx.doi.org/10.1167/iovs.02-0851>.
38. Wingard JB, Romanowski EG, Kowalski RP, Mah FS, Ling Y, Bilonick RA, Shanks RM. 2011. A novel cell-associated protection assay demonstrates the ability of certain antibiotics to protect ocular surface cell lines from subsequent clinical *Staphylococcus aureus* challenge. *Antimicrob Agents Chemother* 55:3788–3794. <http://dx.doi.org/10.1128/AAC.01828-10>.
39. Stella NA, Shanks RM. 2014. Cyclic-AMP inhibition of fimbriae and prodigiosin production by *Serratia marcescens* is strain-dependent. *Arch Microbiol* 196:323–330. <http://dx.doi.org/10.1007/s00203-014-0970-6>.
40. Shanks RM, Stella NA, Hunt KM, Brothers KM, Zhang L, Thibodeau PH. 4 May 2015. Identification of SlpB, a cytotoxic protease from *Serratia marcescens*. *Infect Immun* <http://dx.doi.org/10.1128/IAI.03096-14>.
41. Hume E, Sack R, Stapleton F, Willcox M. 2004. Induction of cytokines from polymorphonuclear leukocytes and epithelial cells by ocular isolates of *Serratia marcescens*. *Ocul Immunol Inflamm* 12:287–295. <http://dx.doi.org/10.1080/0927379490500318>.
42. Zhou R, Zhang R, Sun Y, Platt S, Szczotka-Flynn L, Pearlman E. 2012. Innate immune regulation of *Serratia marcescens*-induced corneal inflammation and infection. *Invest Ophthalmol Vis Sci* 53:7382–7388. <http://dx.doi.org/10.1167/iovs.12-10238>.
43. Kida Y, Inoue H, Shimizu T, Kuwano K. 2007. *Serratia marcescens* serralyisin induces inflammatory responses through protease-activated receptor 2. *Infect Immun* 75:164–174. <http://dx.doi.org/10.1128/IAI.01239-06>.
44. Hazlett LD. 2005. Inflammatory response to *Pseudomonas aeruginosa* keratitis. *Ocul Surf* 3:S139–S141.
45. Willcox MD. 2007. *Pseudomonas aeruginosa* infection and inflammation during contact lens wear: a review. *Optom Vis Sci* 84:273–278. <http://dx.doi.org/10.1097/OPX.0b013e3180439c3e>.
46. Kowalski RP, Romanowski EG, Mah FS, Shanks RM, Gordon YJ. 2010. Topical levofloxacin 1.5% overcomes in vitro resistance in rabbit keratitis models. *Acta Ophthalmol* 88:e120–e125. <http://dx.doi.org/10.1111/j.1755-3768.2010.01897.x>.
47. Mah FS, Romanowski EG, Kowalski RP, Yates KA, Gordon YJ. 2007. Zymar (Gatifloxacin 0.3%) shows excellent Gram-negative activity against *Serratia marcescens* and *Pseudomonas aeruginosa* in a New Zealand White rabbit keratitis model. *Cornea* 26:585–588.
48. Kowalski RP, Romanowski EG, Shanks RM, Mah FS. 2012. The comparison of fluoroquinolones to nonfluoroquinolone antibacterial agents for the prevention of endophthalmitis in a rabbit model. *J Ocul Pharmacol Ther* 28:604–608.
49. Romanowski EG, Mah FS, Yates KA, Kowalski RP, Gordon YJ. 2005. The successful treatment of gatifloxacin-resistant *Staphylococcus aureus* keratitis with Zymar (gatifloxacin 0.3%) in a NZW rabbit model. *Am J Ophthalmol* 139:867–877. <http://dx.doi.org/10.1016/j.ajo.2005.01.021>.
50. Jawad A, Seifert H, Snelling AM, Heritage J, Hawkey PM. 1998. Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates. *J Clin Microbiol* 36:1938–1941.
51. Ramsey M, Hartke A, Huycke M. 2014. The physiology and metabolism of enterococci, p 424–465. In Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), *Enterococci: from commensals to leading causes of drug resistant infection*. Eye and Ear Infirmary, Boston, MA.
52. Frederiksen RF, Paspaliari DK, Larsen TJ, Storgaard BG, Larsen MH, Ingmer H, Palcic MM, Leisner J. 2013. Bacterial chitinases and chitin-binding proteins as virulence factors. *Microbiology* 159:833–847. <http://dx.doi.org/10.1099/mic.0.051839-0>.
53. Sanders NN, Eijssink VG, van den Pangaart PS, Joost van Neerven RJ, Simons PJ, De Smedt SC, Demeester J. 2007. Mucolytic activity of bacterial and human chitinases. *Biochim Biophys Acta* 1770:839–846. <http://dx.doi.org/10.1016/j.bbagen.2007.01.011>.
54. Kirn TJ, Jude BA, Taylor RK. 2005. A colonization factor links *Vibrio cholerae* environmental survival and human infection. *Nature* 438:863–866. <http://dx.doi.org/10.1038/nature04249>.
55. Kawada M, Chen CC, Arihiro A, Nagatani K, Watanabe T, Mizoguchi E. 2008. Chitinase 3-like-1 enhances bacterial adhesion to colonic epithelial cells through the interaction with bacterial chitin-binding protein. *Lab Invest* 88:883–895. <http://dx.doi.org/10.1038/labinvest.2008.47>.
56. Downing KJ, Leslie G, Thomson JA. 2000. Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis* cry1Ac7 and *Serratia marcescens* *chiA* genes in sugarcane-associated bacteria. *Appl Environ Microbiol* 66:2804–2810. <http://dx.doi.org/10.1128/AEM.66.7.2804-2810.2000>.
57. Okay S, Tefon BE, Ozkan M, Ozcengiz G. 2008. Expression of chitinase A (*chiA*) gene from a local isolate of *Serratia marcescens* in Coleoptera-specific *Bacillus thuringiensis*. *J Appl Microbiol* 104:161–170.
58. Ozgen A, Sezen K, Demir I, Demirbag Z, Nalcacioglu R. 2013. Molecular characterization of chitinase genes from a local isolate of *Serratia marcescens* and their contribution to the insecticidal activity of *Bacillus thuringiensis* strains. *Curr Microbiol* 67:499–504. <http://dx.doi.org/10.1007/s00284-013-0395-5>.
59. Khodai-Kalaki M, Aubert DF, Valvano MA. 2013. Characterization of the AtsR hybrid sensor kinase phosphorelay pathway and identification of its response regulator in *Burkholderia cenocepacia*. *J Biol Chem* 288:30473–30484. <http://dx.doi.org/10.1074/jbc.M113.489914>.
60. Gotoh Y, Eguchi Y, Watanabe T, Okamoto S, Doi A, Utsumi R. 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr Opin Microbiol* 13:232–239. <http://dx.doi.org/10.1016/j.mib.2010.01.008>.
61. Lin CS, Horng JT, Yang CH, Tsai YH, Su LH, Wei CF, Chen CC, Hsieh SC, Lu CC, Lai HC. 2010. RssAB-FlhDC-ShlBA as a major pathogenesis pathway in *Serratia marcescens*. *Infect Immun* 78:4870–4881. <http://dx.doi.org/10.1128/IAI.00661-10>.
62. Zolfaghari I, Angus AA, Kang PJ, To A, Evans DJ, Fleiszig SM. 2005. Mutation of *regS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa*, attenuates multiple virulence mechanisms. *Microbes Infect* 7:1305–1316. <http://dx.doi.org/10.1016/j.micinf.2005.04.017>.
63. Tanikawa T, Nakagawa Y, Matsuyama T. 2006. Transcriptional down-regulator HexS controlling prodigiosin and serrawettin W1 biosynthesis in *Serratia marcescens*. *Microbiol Immunol* 50:587–596.