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Increased Proteolytic Activity of *Serratia marcescens* Clinical Isolate HU1848 Is Associated with Higher *eepR* Expression

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Abstract

Serratia marcescens is a global opportunistic pathogen. *In vitro* cytotoxicity of this bacterium is mainly related to metalloprotease serralysin (PrtS) activity. Proteolytic capability varies among the different isolates. Here, we characterized protease production and transcriptional regulators at 37°C of two *S. marcescens* isolates from bronchial expectorations, HU1848 and SmUNAM836. As a reference strain the insect pathogen *S. marcescens* Db10 was included. Zymography of supernatant cultures revealed a single (SmUNAM836) or double proteolytic zones (HU1848 and Db10). Mass spectrometry confirmed the identity of PrtS and the serralysin-like protease SlpB from supernatant samples. Elevated proteolytic activity and *prtS* expression were evidenced in the HU1848 strain through azocasein degradation and qRT-PCR, respectively. Evaluation of transcriptional regulators revealed higher *eepR* expression in HU1848, whereas *cpxR* and *hexS* transcriptional levels were similar between studied strains. Higher *eepR* expression in HU1848 was further confirmed through an *in vivo* transcriptional assay. Moreover, two putative CpxR binding motifs were identified within the *eepR* regulatory region. EMSA validated the interaction of CpxR with both motifs. The evaluation of *eepR* transcription in a *cpxR* deletion strain indicated that CpxR negatively regulates *eepR*. Sequence conservation suggests that regulation of *eepR* by CpxR is common along *S. marcescens* species. Overall, our data incorporates CpxR to the complex regulatory mechanisms governing *eepR* expression and associates the increased proteolytic activity of the HU1848 strain with higher *eepR* transcription. Based on the global impact of EepR in secondary metabolites production, our work contributes to understanding virulence factors variances across *S. marcescens* isolates.

Keywords: *Serratia marcescens*, serralysin, protease, EepR, CpxR

Introduction

Serratia marcescens is a rod-shaped Gram-negative bacterium member of the family *Yersiniaceae*. This bacterium displays a ubiquitous distribution across the soil and aquatic reservoirs. During the last decades, *S. marcescens* has emerged as an important human opportunistic pathogen, causing nosocomial infections in immunocompromised or critically ill individuals, particularly in surgery and intensive care units (Khanna et al. 2013; Cristina et al. 2019). The most commonly reported afflictions caused by this pathogen are keratitis, surgical wound infections, pneumonia, meningitis, and endocarditis (Mahlen 2011). Therapeutic management and control of *S. marcescens* infections is challenging due to its intrinsic resistance to several classes of antibiotics (Gales et al. 2012; Tavares-Carreón et al. 2023). Accordingly, *S. marcescens* was recently included by the World Health Organization (WHO) in the top ten list of resistant pathogens at the intensive care units (Rello et al. 2019).

Besides intrinsic and acquired drug resistance determinants, *S. marcescens* encodes different virulence factors that have been linked with its pathogenic capabilities. Such factors encompass siderophores, hemolysin, lipopolysaccharide, fimbriae, and different proteases

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(Kurz et al. 2003). *S. marcescens* secreted factors have been reported to induce cytotoxic effects on cultured mammalian cells, reducing the viability of cellular monolayers (Carbonell et al. 1997). Proteolytic activity is the primary factor associated with such cytotoxic effects (Marty et al. 2002).

PrtS (also known as PrtA or serralysin), a RTX-toxin family protein, is considered the main secreted metalloprotease among *S. marcescens* isolates, and the *in vitro* cell toxicity induced by this bacterium is mainly attributed to PrtS (Marty et al. 2002; Shanks et al. 2015). The optimal production of PrtS, as well as different metabolites, including prodigiosin pigment, is reported at low temperatures (< 30°C), at post-exponential growth (Williams et al. 1971; Petersen and Tisa 2012). In this scenario, thermoregulation of *prtS* through the two-component system (TCS) CpxAR is hypothesized to prevent serralysin cytotoxicity toward mammalian cells during colonization of regulated-body-temperature hosts (Bruna et al. 2018).

Depending on the *Serratia* isolate, up to four additional *prtS* homologues genes can be found encoded within their genome and referred to as serralysin-like proteases (Slps) from B to E. These Slps are considered necessary for full cytotoxicity (Shanks et al. 2015). Notwithstanding, their production and specific contribution during *S. marcescens* pathogenesis is poorly understood. In addition, serralysin homologues are encoded across different genera, including *Pseudomonas*, *Erwinia,* and *Dickeya* (Stocker et al. 1995). A type I secretion system (T1SS) is commonly employed by bacteria secreting RTX-toxins (Spitz et al. 2019). In *S. marcescens*, expression of *lipBCD* (T1SS) along with secondary metabolites and degradative enzymes (including metalloprotease genes) are positively regulated by the TCS, EepRS (Brothers et al. 2015; Stella et al. 2017). Regulation of response regulator EepR and its downstream induced phenotypes is tightly controlled. For instance, HexS (a LysR family regulator) negatively regulates the expression of *eepR* and secondary metabolites (including *prtS*) (Tanikawa et al. 2006; Shanks et al. 2017). The cAMP receptor protein (CRP) also limits pigment and protease production (Kalivoda et al. 2010; Shanks et al. 2013), presumably through the direct transcription inhibition of *eepR* (Stella et al. 2015).

Proteolytic activity varies significantly among *S. marcescens* isolates (Shanks et al. 2015). Accordingly, our group has previously described from a collection of over 180 *S. marcescens* clinical isolates that strains from the respiratory tract are predominantly characterized for a higher proteolytic activity (Gonzalez et al. 2020a). Here, we aimed to analyze this phenotype by using two *S. marcescens* isolates from bronchial secretions at different Mexican health care institutions, SmUNAM836 (Sandner-Miranda et al. 2016), and HU1848 (Gonzalez et al. 2020b). As a reference strain, the well-characterized insect pathogenic model, *S. marcescens* Db10 was included (Flyg et al. 1980; Kurz et al. 2003). Evaluations were achieved at 37°C, a temperature known to restrict protease due to the activation of the response regulator CpxR (Bruna et al. 2018). Obtained data provide further evidence supporting that contrasting proteolytic activity between the evaluated *S. marcescens* strains correlates with the *eepR* expression levels. It also incorporates CpxR as a direct negative regulator of *eepR*.

Experimental

Materials and Methods

Bacterial strains and growth conditions. *S. marcescens* SmUNAM836 (Sandner-Miranda et al. 2016); HU1848 (Gonzalez et al. 2020b); and Db10 (Flyg et al. 1980), and *Escherichia coli* TOP-10; BL21 DE3 pLysS; and BW25412 (Haldimann and Wanner 2001), were routinely cultured in Lysogeny Broth (LB), 1% tryptone, 0.5% yeast extract and 1% NaCl. When required, LB media were supplemented with 150 µg/ml ampicillin; 50 or 150 µg/ml kanamycin; 30 or 100 µg/ml chloramphenicol, for *E. coli* or *S. marcescens* strains, respectively.

In-frame deletion in *cpxR* was constructed by cloning *cpxR* flanking regions into pTOX3 suicide plasmid (Lazarus et al. 2019). Upstream and downstream (600–650 bp) *cpxR* regions were PCR amplified with the appropriate oligonucleotides (Table I), using as template chromosomal DNA of *S. marcescens* HU1848. PCR products were cloned by Gibson Assembly® (New England BioLabs, USA) in a pTOX3 plasmid previously digested with *Eco*RV (New England BioLabs, USA). Assembly reaction was electroporated into *E. coli* BW25412 and resulting colonies were screened by PCR using primers scTOXF and scTOXR (Table I). Correct amplification was verified by sequencing at IPICYT (Instituto Potosino de Investigación Científica y Tecnológica, Mexico). Resulting construction pTOX3cpxR was electroporated into *S. marcescens* HU1848 and PCR verified plasmid integration. A single positive colony was grown in LB 2% glucose to an OD_{600} of 0.2, washed twice with M9 minimal medium (BD Difco™, USA), and plated on M9-agar supplemented with 2% rhamnose. The resulting double-crossover mutants were identified by PCR using primers XRscFw and XRscRv (Table I).

Protease activity. Quantitative determination of protease activity was measured from spent culture supernatants using the colorimetric substrate azocasein (Megazyme, Ireland) and following fabricant recommendations. Briefly, bacteria were grown for 18 h at 37°C with aeration in LB, and cleared supernatants

Table I
Oligonucleotides used in this study.

Primer	5'–3' primer sequence	Purpose
XRQFw	TGGAAGCCATGCATAAACTG	Internal pair for *cpxR*
XRQRv	TACGCTGCTGATGTTTCTGG	
16SQFw	GAGCAAGCGGACCTCATAAAG	Internal pair for 16S
16SQRv	TGCGGTTGGATTACCTCCT	
ERQFw	GGATTGGAAAACGTCAGCATG	Internal pair for *eepR*
ERQRv	GCCACGAAAAAGATGGCATC	
HSQFw	CTTCCAGCAGATCGACCATC	Internal pair for *hexS*
HSQRv	AGATCCTGCGCTTTAACGAC	
SDQFw	CGCGATCCAAAAATTGTACG	Internal pair for *slpD*
SDQRv	TCGTTCAGGTTGATCATCTG	
PSQFw	GACCTGGTACAACGTCAAC	Internal pair for *prtS*
PSQRv	GTAGCTCATCAGGCTGAAC	
PREcoF	CCCTGAATTCCGTTTTTATTTGCGGCTG	Pair for *eepR* promoter cloning into pSEVA246
PRXbaR	TGGGTCTAGATTGTTATCCATTTGTTCCTTCG	
scSEVAF	AGCGGATAACAATTTCACACAGGA	Pair for pSEVA- based constructions screening
scSEVAR	CTTTCGGGAAAGATTTCAACCTGG	
scETFw	CCCTCAAGACCCGTTTAGAG	Pair for pET- based constructions screening
scETRv	CTCTTCCGAGGTGAAAACCG	
xRNdeF	CGCGCATATGAACAAGATTCTGTTAG	Pair for *cpxR* amplification
xRBamR	TTTGGATCCAAACTGTTGATCATGTTGC	
CRPFw	CCTGGTGCCGCGCGGCAGCCATGTTCTCGGCAAACCGCAAAC	Pair for *crp* amplification
CRPRv	CTGTCCACCAGTCATGCTAGCCATTAACGGGTGCCGTAGACG	
HexSFw	CCTGGTGCCGCGCGGCAGCCACATGACAACTGCAAATCGTCC	Pair for *hexS* amplification
HexSRv	CTGTCCACCAGTCATGCTAGCCACGTTATTCTTCTTCGTCCAC	
PeRFw1	CAATAAAAAACCGGGACCC	Forward for *eepR* promoter (–358 nt from *eepR* start)
PeRFw2	GCAGTCCRAGCGATGTG	Forward for *eepR* promoter (–271 nt from *eepR* start)
PeRRv2	TTTCYGCTGAAAAAGCCAC	Reverse primer for *eepR* promoter (+45 nt from *eepR* start)
PeRRv1	TTGTTATCCATTTGTTCCTTCG	Reverse primer for *eepR* promoter (-11 nt from *eepR* start)
recAFw	CAAGGCGAATGCCTGTAACT	Pair for EMSA negative control (internal for *recA*)
recARv	GAGGATAGGCGCCACATAAA	
UPxRFw	GGGTTTTTTCGCTGATCACGTACGATGCGCTGCTGATGTTTCTGG	Pair for *cpxR* upstream region
UPxRRv	GTTGCGCCAGCAGATACAGCAGCGAGGTCAACTCGCGGTC	
DWxRFw	GACCGCGAGTTGACCTCGCTGCTGTATCTGCTGGCGCAAC	Pair for *cpxR* downstream region
DWxRRv	GTACACCATGTGCACCGGTTCGAAGATGGTGACGATCAGCAGCAG	
scTOXF	CGCGACGGTTTCTTACAGTG	Pair for pTOX3- based construction screening
scTOXR	GCTTCCCGGTATCAACAGAG	
XRscFw	CCAGAAATTTGTTGCTCCATC	Pair for *cpxR* deletion strain screening
XRscRv	GGTCGGAACATCAGGTTGAT	

Restriction endonuclease sites *Eco*RI, *Xba*I, *Nde*I and *Bam*HI incorporated in the oligonucleotide sequences are underlined.

were obtained by centrifugation and filtration through a 0.22 µm pore filter. An aliquot of 125 µl of supernatant was transferred into a clean tube, mixed 1:1 with azocasein (2% w/v), and incubated for 30 min at 30°C. Reactions were stopped by adding 750 µl of trichloroacetic acid solution (5% w/v). Tubes were centrifuged at 8,000 × *g* for 5 min, and 50 µl of supernatant were mixed with 150 µl of 1 N NaOH into a 96-well plate. The liberation of azo dye was measured at 440 nm with a plate reader; values were normalized to the original

culture growth at OD_{600}. Three independent biological replicates were performed per strain.

Supernatant proteins precipitation. For secreted protein precipitation, 100 ml of LB cultures (16 h at 37°C) of *S. marcescens* SmUNAM836, HU1848, or Db10 were centrifuged at 12,000 × *g* for 20 min and filtered through a 0.22 µm filter. Cleared supernatants were then transferred to a beaker, and ammonium sulfate was added slowly with constant stirring. After reaching 70% ammonium sulfate saturation, samples were incubated with stirring at 4°C for 30 min and centrifuged at 12,000 × *g* for 40 min at 4°C. Supernatants were discarded, and pellets were resuspended with 200 µl of PBS and dialyzed overnight against 1.5 l of ultra-pure water. Protein concentration was determined by the BradFord ULTRA (BioRad, USA). Precipitated proteins were analyzed by SDS-PAGE. For protein conservation, glycerol was added (10% final concentration), and samples were stored at –20°C.

Gelatin zymography. For in gel activity, 1µg of supernatant precipitated proteins was mixed with non-reducing SDS-loading buffer and separated by 10% SDS-PAGE in gels co-polymerized with 0.15% (w/v) gelatin. Following electrophoresis, gels were washed three times with washing buffer (50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 0.02% NaN_3, 10 µM $ZnCl_2$, 1.25% Triton X-100). Then, gels were incubated with developing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3, 10 µM $ZnCl_2$, 0.02% Brij-35) for 1 h at 30°C, when required, 50 mM of EDTA was added to washing and developing buffers. After incubation gels were stained with Coomassie brilliant blue. Zones of proteolysis were detected after de-staining with water.

Proteomic analysis. SDS-PAGE excised bands of supernatant samples of *S. marcescens* HU1848 and SmUNAM836 grown at 37°C were submitted to the LUP-UNAM proteomic facility in Mexico. Mass spectrometric data were obtained using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Inc., USA) matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)/TOF spectrometer. The resulting mass spectra were used for identifying the proteins by the Mascot search engine using the Uniprot-*Serratia m* database with the software Proteome Discoverer 1.4.

Transcriptional analysis. Overnight LB cultures of *S. marcescens* strains were subcultured into 8 ml of fresh LB media to a starting $OD_{600} = 0.05$ and grown at 37°C with shaking until they reach $OD_{600} = 1.0$. For Δ*cpxR* strain complementation (see below) 1 mM arabinose was added to the culture. The RNA was extracted using the Total RNA Purification Kit (Jena Bioscience, Germany) following manufacturer recommendations. Then, purified RNA was treated with DNase I (Jena Bioscience, Germany) and later with a gDNA Removal Kit (Jena Bioscience, Gemany). RNA concentration was measured using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Inc., USA). The absence of chromosomal DNA contamination was verified by qRT-PCR of an internal fragment of the the16S rRNA gene (Table I). Synthesis of cDNA was performed using SuperScript™ II Reverse Transcriptase (Invitrogen™, USA). The qRT-PCR evaluation was performed using qPCR GreenMaster (Jena Bioscience, Germany) following fabricant recommendations in a Rotor-Gene Q (QIAGEN, Germany) and using internal oligonucleotides for the indicated genes (Table I). The qRT-PCR analysis was achieved using the $2^{-\Delta CT}$ method (Schmittgen and Livak 2008), and the C_T value of each gene of interest was normalized to the C_T value of the 16S rRNA housekeeping control gene of individual strains.

Evaluation of *eepR* promoter transcriptional activity. A region consisting of 415 nucleotides upstream of the *eepR* start codon was amplified by PCR using genomic DNA of HU1848 or SmUNAM836 strains and primers PREcoF and PRXbaR (Table I). PCR products were double-digested with *Eco*RI and *Xba*I (Invitrogen™, USA) and ligated with T4 DNA ligase (Roche, Switzerland) into a similarly digested pSEVA246 plasmid (Martínez-García et al. 2020). Ligation reactions were electroporated into *E. coli* TOP-10. The resulting colonies were screened by PCR using primers scSEVAF and scSEVAR (Table I). Correct amplification of each construction (p*eepR*$_{HU1848}$ and p*eepR*$_{SmUNAM}$) was verified by sequencing at IPICYT. Then, plasmids p*eepR*$_{HU1848}$ and p*eepR*$_{SmUNAM}$ were electroporated into *S. marcescens* HU1848 or SmUNAM836. Luminescence activity was tested in LB cultures at 37°C as previously reported (Gonzalez-Montalvo et al. 2021) using a GloMax® (Promega, USA) plate reader.

Cloning of *cpxR, crp,* and *hexS* genes and protein purification. The *cpxR*, *crp* and *hexS* genes were PCR amplified with the appropriate oligonucleotides (Table I), using as template chromosomal DNA of *S. marcescens* HU1848. Purified PCR product of *cpxR* was double digested with *Nde*I and *Bam*HI (Invitrogen™, USA) restriction enzymes and ligated into a similarly digested pET19b (Novagen®, Merck KGaA, Germany) plasmid using T4 DNA Ligase (Invitrogen™, USA). The purified PCR products of *crp* and *hexS* were assembled into a *Nde*I digested pET28 plasmid using Gibson assembly (New England Biolabs, USA). Ligation or assembly reactions were electroporated into *E. coli* TOP-10. The resulting colonies were screened by PCR using primers scETFw and scETRv (Table I). At least one positive colony of each construction (pET19cpxR, pET28crp, or pET28hexS) was sequenced as described above. For Δ*cpxR* strain complementation, plasmid pET19cpxR was double digested with *Xba*I and *Hind*III, and the resulting *cpxR* gene was purified by gel extraction and ligated into a similarly digested pBAD33 plas-

mid yielding to pBADcpxR. For gene over-expression, pET-based constructions were mobilized into *E. coli* BL21 DE3 pLysS. The induction of recombinant proteins was achieved using LB cultures (200 ml at 37°C), supplemented with kanamycin or ampicillin; once the culture reached an $OD_{600} = 0.5$, 0.3 mM of IPTG was added, and incubation continued during 4 hr. Bacteria were harvested by centrifugation, and resulting pellets were resuspended in binding buffer (BB; 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl) containing a protease inhibitor cocktail (Jena Bioscience, Germany) and lysed by sonication. Bacterial lysates were centrifuged at 15,000 × g for 60 min at 4°C. The soluble fractions were applied to a Ni-NTA resin (QIAGEN, Germany) for 30 min at 4°C. After extensive washing with BB containing 40 mM imidazole, the proteins were eluted with 200 µl of BB containing 300 mM. Purified recombinant proteins were dialyzed for 6 h at 4°C against 1 l of TND buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT)). Purified proteins were analyzed by SDS-PAGE and protein concentration was determined by the Bradford ULTRA (BioRad, USA).

Electrophoretic gel mobility shift assay (EMSA). The regulatory region of *eepR* was PCR amplified using the template chromosomal DNA of *S. marcescens* HU1848 or SmUNAM836, and the indicated oligonucleotide pairs (Table I). Fifty nanograms of purified PCR products were mixed with dialyzed His-tagged CRP (0.05–0.4 µM); or HexS (0.5–4 µM); or CpxR (1 µM) in a buffer containing 40 mM HEPES (pH 8.0), 8 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 0.05% Igepal, 10% glycerol and 0.1 mg/ml BSA. Reaction mixtures were incubated for 30 min at 30°C and subjected to electrophoresis on 5% PAGE native gels at 4°C (for CRP reaction buffer and polyacrylamide gel were supplemented with 50 and 100 µM cAMP, respectively; and for CpxR, when indicated the protein was pre-incubated with 25 mM acetyl phosphate at 30°C for 30 min before incubation with the DNA). Gels were visualized in a UVP GelStudio (Analytik Jena GmbH + Co. KG, Germany) after staining with 0.1% of ethidium bromide. Each experiment was repeated at least two times, yielding similar results.

Statistical analysis. Student's *t*-test or Mann-Whitney *U*-tests were used for statistical analyses using the GraphPad Prism 8 software (www.graphpad.com) with significance set to $p < 0.05$.

Results and Discussion

***In-gel* proteolytic activity profile of *S. marcescens* secreted proteins.** To better characterize the proteolytic activity displayed by *S. marcescens* strains, we examined the electrophoretic patterns of their secreted proteins in-gel zymography (Fig. 1). Culture supernatants of strains HU1848, SmUNAM836, and Db10 grown at 37°C were filtered and concentrated through ammonium sulfate precipitation (as described in Material and Methods). Previous analysis of concentrated *S. marcescens* supernatants from saturated cultures reported null or rare bacterial lysis (Di Venanzio et al. 2014; Lazzaro et al. 2017). An equivalent amount of protein was separated by SDS-PAGE and stained with Coomassie brilliant blue. In agreement with previous reports (Brothers et al. 2015), protein bands above the 48-kDa-protein marker were observed in the supernatant samples of the three *S. marcescens* strains (Fig. 1A). Clear zones due to hydrolysis of the substrate (gelatin) appeared in the zymogram at the equivalent migration area (above 48 kDa) (Fig. 1B). Moreover, a slightly higher (~55 kDa) second gelatin degradation zone was observed in supernatant samples of HU1848 and Db10 strains, but not in SmUNAM836 (Fig. 1B). *Serratia* zymography pattern is poorly defined, nonetheless obtained results with HU1848 and Db10 resemble zymogram data of pioneer work in *S. marcescens* strain BG (Lyerly and Kreger 1979). In contrast, a unique proteolytic zone (similar to SmUNAM836) was also described in the supernatant of the environmental strain AD-W2 (Chander et al. 2021).

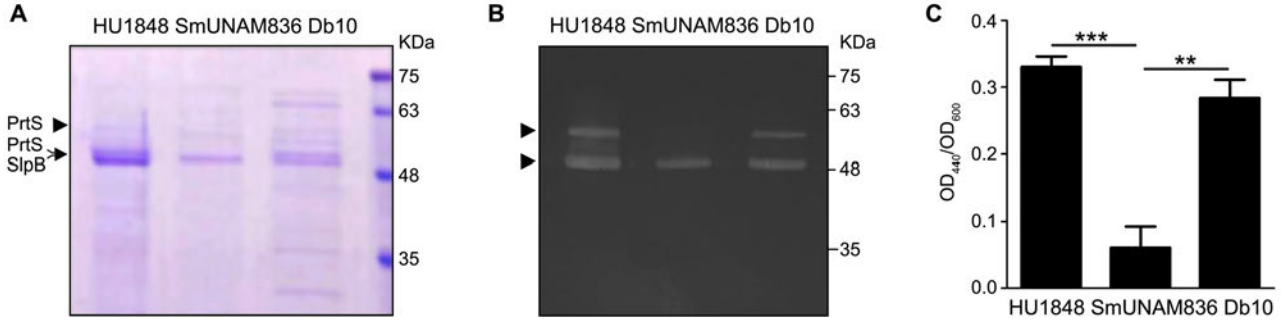

Fig. 1. Zymography and proteolytic activity of *Serratia marcescens* strains.

A) SDS-PAGE of extracellular proteins precipitated by ammonium sulfate from bacterial cultures at 37°C (Coomassie stain). Identified proteins by mass spectrometry are indicated at the left; B) Zymography following SDS-PAGE using gelatin as substrate from supernatant cultures grown at 37°C. Arrowheads indicate gelatin degradation areas; C) Digestion of azocasein by normalized filtered supernatants. Graphs represent the mean ± SEM from three independent experiments (**$p < 0.01$, ***$p < 0.001$).

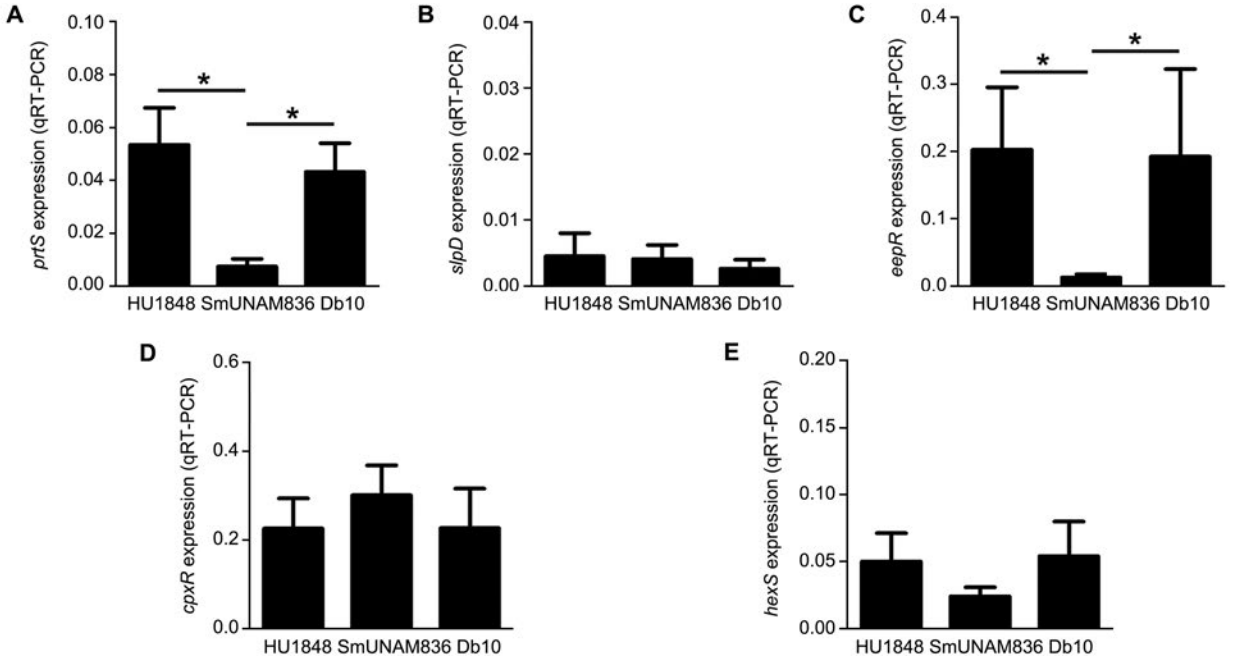

Fig. 2. Transcriptional analysis of *prtS* and regulator genes in *Serratia marcescens* HU1848, SmUNAM836 and Db10. qRT-PCR analysis of gene expression of A) *prtS*, B) *slpD*, C) *eepR* D) *cpxR*, and E) *hexS*. RNA was extracted from bacterial cultures at 37°C. The mRNA levels were normalized to the 16S rRNA gene. Relative expression was calculated by $2^{-\Delta CT}$ method Means ± SEM from three independent experiments are shown (*$p < 0.05$).

In order to correlate the protease identity with observed substrate degradation zones, SDS-PAGE-separated bands of HU1848 and SmUNAM836 were excised and subjected to LC-MS/MS. As expected, the protein migrating just above the 48 kDa marker was identified as PrtS (52.2 kDa) in both HU1848 and SmUNAM836, with a peptide coverage of 51% and 38%, respectively (Fig. S1). In addition, SlpB (50.3 kDa) was also identified in the same sample (~48 kDa) of strain HU1848, albeit with low coverage (12%) (Fig. S1). This was in agreement with a report of strain K904, a keratitis isolate, indicating an equal in-gel migration of PrtS and SlpB, as well as lower protein levels of SlpB (Shanks et al. 2015). On the other hand, the SmUNAM836 genome does not encode SlpB.

Moreover, in the 55 kDa hydrolytic zone of HU1848, PrtS was also identified by mass spectrometry with a coverage of 43%. Based on *prtS* gene sequence, a precursor form of PrtS with an extended N-terminus is considered to occur (Braunagel and Benedik 1990). In addition, the same situation of two closely migrating proteases identified as PrtS after amino-terminal sequencing was previously described in *S. marcescens* ATCC® 25419™ (Schmitz and Braun 1985). Synthesis of PrtS as a higher mass pre-protein is also supported by two independent works showing that expression of *prtS* in *E. coli* cells results in a slightly larger molecular weight enzyme than the PrtS detected in *Serratia* (Nakahama et al. 1986; Braunagel and Benedik 1990). No detection of PrtS isoform in SmUNAM836 by zymography might result from nucleotide differences upstream to the putative *prtS* start codon (data not shown).

High proteolytic activity of *S. marcescens* HU1848 correlates with elevated *prtS* and *eepR* expression. To continue characterizing the protease production of *S. marcescens* isolates, their proteolytic activity was assessed through azocasein hydrolysis using supernatant samples from cultures at 37°C. Obtained results showed a significantly higher (5.4 fold) proteolytic activity of HU1848 compared to SmUNAM836 (Fig. 1C). Protease activity of HU1848 was comparable to the entomopathogen strain Db10 (Fig. 1C). Then, to correlate protease activity determination with protease gene expression, we extracted RNA from the three strains grown at 37°C and achieved qRT-PCR evaluations (Fig. 2). In agreement, *prtS* transcript levels (Fig. 2A) were significantly elevated in HU1848 (7.24-fold) and in Db10 (5.85-fold) compared to SmUNAM836 ($p = 0.028$). In contrast, no significant differences were obtained in transcript levels of the protease *slpD* (Fig. 2B). PrtS is defined as the primary contributor to *S. marcescens* proteolytic activity (Bruna et al. 2018). Accordingly, the deletion of *slpB* only showed a slight reduction in the proteolytic capability of the keratitis isolate K904 (Shanks et al. 2015). Thus, we associated the reduced azocasein degradation in SmUNAM836 with lower production of PrtS rather than with the lack of SlpB.

To date, *prtS* expression has been reported to be directly influenced by transcriptional regulators *cpxR*, *eepR*, and *hexS*. (Shanks et al. 2017; Bruna et al. 2018).

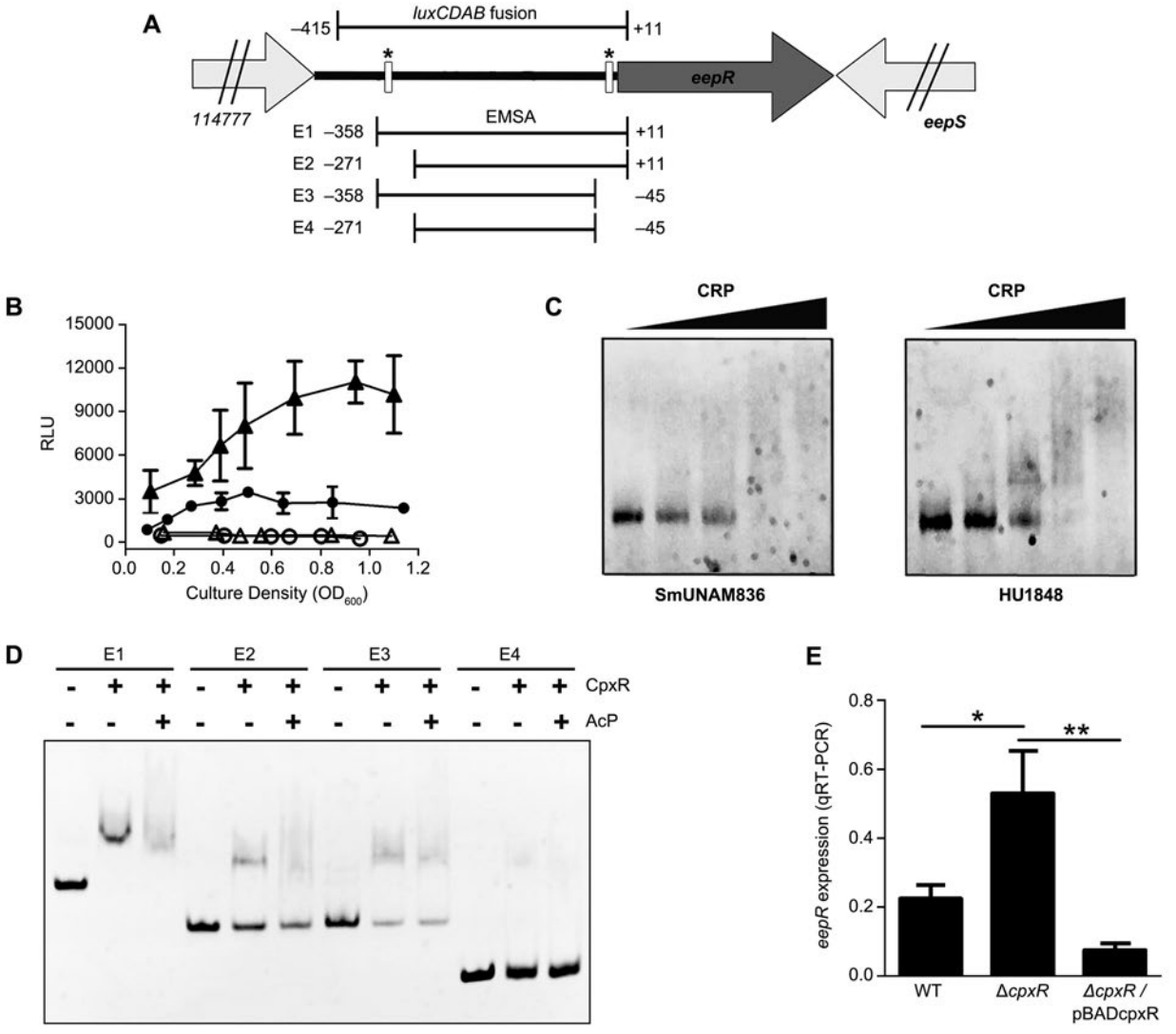

Fig. 3. Differential *eepR* promoter expression and direct regulation of *eepR* by CpxR.

A) Scheme representation of the *Serratia marcescens* region encoding *eepRS*. DNA fragments employed in transcriptional fusion or in EMSA evaluations are depicted. White rectangles with asterisk indicate the two predicted CpxR binding sites. All nt positions are referred to *eepR* start codon; B) *eepR* promoter activity expressed as RLU during bacterial culture grown at 37°C. Filled and open triangles HU1848 carrying p*eepR*$_{HU1848}$ or pSEVA26, respectively. Filled and open circles SmUNAM836 carrying p*eepR*$_{SmUNAM}$ or pSEVA26, respectively; C) EMSA using CRP (0, 0.05, 0.1, 0.2, 0.4 μM) and DNA region E1 amplified from SmUNAM836 or HU1848; D) EMSA using CpxR (0, 0.5, 1, 2, 3 μM) and the four DNA fragments (E1-E4) containing *eepR* upstream sequence of strain HU1848, reactions pre-incubated with acetyl phosphate are indicated (AcP).

E) qRT-PCR analysis of *eepR* expression from the *S. marcescens* strain HU1848 (WT), Δ*cpxR* strain and Δ*cpxR* carrying pPBADcpxR plasmid. Relative expression was calculated by $2^{-\Delta CT}$ method. Means ± SEM from three independent experiments are shown (**$p < 0.01$, *$p < 0.05$).

Therefore, we decided to compare the transcript levels of these three regulators. Our data indicated a 16.8 and 15.9-fold *eepR* expression in HU1848 and Db10 strains, respectively, related to SmUNAM836 ($p = 0.019$ and $p = 0.015$, respectively) (Fig. 2C). In contrast, no significant differences were determined in transcript levels of *cpxR* or *hexS* (Fig. 2D and 2E). In order to corroborate the higher *eepR* transcription determined in HU1848, we fused the *eepR* upstream region of HU1848 and SmUNAM836 (Fig. 3A) to the promoterless *luxCDABE* operon in plasmid pSEVA246 (Martínez-García et al. 2020). The *S. marcescens* strains carrying *eepR* transcriptional reporter were grown at 37°C and relative luminescence was determined at different time points (Fig. 3B). In agreement with our qRT-PCR data, luminescence values showed a significantly elevated *eepR* expression in HU1848 strain throughout culture growth compared to SmUNAM836 ($p < 0.05$) (Fig. 3B). Control plasmids indicated negligible luciferase basal activity in both strains (Fig. 3B), confirming the higher transcriptional activity of *eepR* in strain HU1848.

Our data suggest that the higher proteolytic activity, displayed by HU1848 and Db10 strains, is at least partially associated with elevated *eepR* expression. Thus, we looked at the *eepR* upstream sequences. Several substitutions were noticed, including two at the CRP binding site and six at the immediate upstream region (Fig. S2). Comparison with different *S. marcescens* isolates revealed

that these nucleotide changes are shared by several representative strains (Fig. S2). Moreover, two conserved putative CpxR binding sites were identified within *eepR* regulatory region (Fig. 3A and S2).

CRP has been described as an *eepR* repressor (Stella et al. 2015; Shanks et al. 2017); based on the position of the sequence differences within the *eepR* regulatory region of the studied strains, we cloned *crp* and purified a recombinant CRP (Fig. S3A). However, when we evaluated the interaction of CRP with the *eepR* upstream sequence of SmUNAM836 or HU1848 a similar migration was observed (Fig. 3C). A comparable result was obtained using a recombinant HexS (Fig. S4). Interaction specificity was corroborated using a DNA fragment lacking the predicted CRP binding site or an unrelated DNA fragment (Fig. S4). According to these results, the differences in *eepR* expression of studied strains are unlikely as a consequence of CRP or HexS impaired recognition of *eepR* regulatory region. Nonetheless, a more detailed genetic analysis is needed to assess if the *eepR* upstream sequence differences (Fig. S2) modify the affinity of CRP or HexS (or another transcriptional regulator), resulting in differential expression of *eepR* in *S. marcescens* strains.

In addition, other factors not necessarily defined by the *eepR* regulatory sequence could indirectly impact *eepR* transcriptional activity; for instance, unlike SmUNAM836, strains Db11 and HU1848 lack the *luxI* gene responsible for *N*-acyl-L-homoserine lactone (AHL) molecule, implicated in quorum sensing (QS) signaling (Sakuraoka et al. 2019). In this regard, QS signaling in the environmental species *Serratia liquefaciens* MG1 and *Serratia proteamaculans* B5a was found to be involved in the regulation of the T1SS *lipBCD* and consequently affecting the exoenzymes secreted through this system (Riedel et al. 2001; Christensen et al. 2003), a phenotype that resembles a *S. marcescens eepR* deletion strain (Brothers et al. 2015; Stella et al. 2017). Nonetheless, a direct impact of QS molecules over *eepR* expression in AHL producer *Serratia* strains remains to be investigated.

CpxR interacts with the *eepR* regulatory region and negatively regulates its expression. Two putative CpxR binding sites (greatly conserved between *S. marcescens* species) were noticed within *eepR* upstream sequence (Fig. 3A and S2). To assess whether CpxR directly contributes to the expression of *eepR* we purified a recombinant CpxR protein (Fig. S3C). It was implemented in EMSA evaluations using different DNA fragments containing or not the putative CpxR binding sequences (Fig. 3A and 3D). A clear interaction of CpxR to *eepR* upstream sequence was noticed, particularly when both binding sites were included (Fig. 3D; E1 lane). CpxR interaction decreased when a single site was present (Fig. 3D; E2 and E3 lanes), indicating that CpxR binds to both motifs. Lastly, CpxR interaction appears specific since it was lost when a fragment lacking both recognition sequences was tested (Fig. 3D; E4 line). In addition, CpxR binding was similar in the absence of acetyl phosphate, suggesting that CpxR can interact *in vitro* with *eepR* regulatory region regardless of its phosphorylation (Fig. 3D).

Moreover, to evaluate the role of CpxR over transcriptional regulation of *eepR*, a *cpxR* deletion strain was constructed in the HU1848 background. Total RNA was extracted from cultures at 37°C, a temperature at which CpxR became more active (Bruna et al. 2018). We found that *eepR* expression was 2.3-fold elevated in the *cpxR* mutant (Fig. 3E, $p = 0.03$) compared to the parental strain. Also, the *eepR* mRNA values were lowered when the *cpxR* mutant was carrying the *cpxR* gene *in trans* (Fig. 3E). Therefore, our data confirms a direct role of CpxR operating as a negative regulator of *eepR*.

According to its direct positive role in producing different exoenzymes, including PrtS (Stella et al. 2015), EepR is considered a key regulator of host-pathogen interactions promoting proinflammatory response (Brothers et al. 2021). In this scenario, the induction of *prtS* might further contribute to bacterial airway pathogenicity through the activation of the epithelial sodium channel (ENaC) (Butterworth et al. 2014). Also, experimental models have shown that intranasal administration of PrtS provokes a deleterious impact, leading mouse lungs to be markedly susceptible to viral infection (Akaike et al. 1989). Nonetheless, elucidating an early cytotoxicity might accelerate bacterial clearance, preventing tissue colonization. Thus, negative thermoregulation of *prtS* by CpxR (at 37°C) is believed to grant warm-blooded hosts colonization and to regulate *S. marcescens* biofilm community (Bruna et al. 2018).

In summary, we have shown here that elevated expression of EepR in the respiratory isolate HU1848 is associated with increased *prtS* levels and concomitant proteolytic activity. Moreover, we described that CpxR directly binds to the upstream region of *eepR* and inhibits the expression of this positive regulator. However, further research is needed to understand the molecular mechanisms relieving the *eepR* repression on particular *S. marcescens* isolates.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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