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Suppressor analysis of *eepR* mutant defects reveals coordinate regulation of secondary metabolites and serralysin biosynthesis by EepR and HexS

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Abstract

The EepR transcription factor positively regulates secondary metabolites and tissue-damaging metalloproteases. To gain insight into mechanisms by which EepR regulates pigment and co-regulated factors, genetic suppressor analysis was performed. Suppressor mutations that restored pigment to the non-pigmented Δ*eepR* mutant mapped to the *hexS* ORF. Mutation of *hexS* also restored haemolysis, swarming motility and protease production to the *eepR* mutant. HexS is a known direct and negative regulator of secondary metabolites in *Serratia marcescens* and is a LysR family regulator and an orthologue of LrhA. Here, we demonstrate that HexS directly controls *eepR* and the serralysin gene *prtS*. EepR was shown to directly regulate *eepR* expression but indirectly regulate *hexS* expression. Together, these data indicate that EepR and HexS oppose each other in controlling stationary phase-associated molecules and enzymes.

INTRODUCTION

In stationary phase, the Gram-negative bacterium and opportunistic pathogen *Serratia marcescens* synthesizes a number of secondary metabolites and secreted enzymes. Generation of these factors is highly regulated by a number of transcription factors including negative regulators CopA [1], CRP [2], HexS [3, 4], RssAB [5] and SpnR [6] and positive regulators EepR [7, 8], PigP [3] and SmaI [9].

The EepR putative response regulator is a direct positive regulator of several compounds including the biologically active pigment prodigiosin, the antibiotic biosurfactant serratamolide and the cytotoxic metalloprotease serralysin (PrtS) [7, 8]. The *eepR* gene is also important in positive regulation of chitinases and chitin binding protein Cbp21, as well as other proteins such as the SlpB protease and S-layer protein SlaA [7]. EepR-like regulators have been found in other medically relevant organisms including *Burkholderia* species [10]. The coordinated interplay between EepR and other transcriptional regulators that govern secondary metabolites and virulence factors has not been determined.

In this study, suppressor analysis was used to gain insight into the regulatory network of the EepR transcription factor. Transposon mutations that restored pigmentation to a Δ*eepR* mutant mapped to the *hexS* transcription factor and upstream of the *eepR* ORF. Subsequent analysis supports that HexS directly binds to and inhibits *eepR* expression and that EepR inhibits *hexS* expression. Together, the data presented here suggest that EepR and HexS are key regulators that oppose one another in control of secondary metabolites and the cytotoxic metalloprotease serralysin.

METHODS

Microbiological growth conditions and media

Escherichia coli and *S. marcescens* strains are listed in Table 1 and were grown in lysogeny broth (LB) [11, 12] at 30 °C. Growth in liquid medium was performed with aeration using a tissue culture roller (TC-7). Swarming motility plates were composed of LB with 0.6 % agar, and swimming motility plates were LB with 0.3 % agar. Haemolysis detection plates consisted of tryptic soy agar with 5 % sheep erythrocytes. Antibiotics used were gentamicin at 10 μg ml^{-1}, kanamycin at 50–100 μg ml^{-1} and tetracycline at 10 μg ml^{-1}.

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Abbreviations: EMSA, electrophoretic mobility shift assay; LB, lysogeny broth; MBP, maltose binding protein; qRT-PCR, quantitative reverse transcriptase PCR.

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Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source*
Saccharomyces cerevisiae		
InvSc1	Uracil auxotroph for *in vivo* cloning	Invitrogen
E. coli		
SM10 λpir	*thi thr leu tonA lacY Sup.E recA*::*RP4-2Tc*::*Mu pir*	[19]
S17-1 λpir	*thi pro hsdR hsdM*$^{+}$ Δ*recA RP4-2*::*TcMu-Km*::*Tn7 pir*	[19]
EC100D *pir*-116	Cloning strain	Epicentre
S. marcescens		
PIC3611	Wild-type 'parental strain'	Presque Isle Cultures
CMS2922	PIC3611 Δ*hexS*	[17]
CMS2097	PIC3611 Δ*eepR*	[8]
CMS2204	PIC3611 Δ*eepR* Δ*hexS*	This study
Nima	Wild-type 'parental strain'	Pryce Haddix
CMS2089	Nima Δ*eepR*	[8]
CMS2320	Nima Δ*eepR* Δ*hexS*	This study
CMS3125	CMS2922 with pMQ294 integrated at *hexS*	[17]
Plasmids		
pMQ131	$_{ori}$pBBR1 *aphA-3* oriT *URA3 CEN6/ARSH4*	[18]
pMQ236	$_{ori}$R6K *nptII rpsL oriT URA3 CEN6/ARSH4 I-SceI* site	[18]
pMQ240	$_{ori}$pSC101^{ts}*aacC1 oriT* P_{lac}*-I-SceI URA3 CEN6/ARSH4*	[18]
pMQ248	pMQ131 with P_{flhDC}*-lacZ*	[3]
pMQ292	pMQ131 with *hexS*	[17]
pMQ294	pMQ236 with *hexS* wild-type allele	[17]
pMQ296	pMQ236 with *hexS*-Δ1 mutant allele	[17]
pMQ361	pMQ131 with P_{nptII}*-tdtomato*	[21]
pMQ407	pMQ131 with *lrhA* from *E. coli*	This study
pMQ412	pMQ361 with P_{eepR}*-tdtomato*	[8]

*Invitrogen, Waltham, MA, USA; Epicentre Biotechnologies, Madison, WI, USA; Presque Isle Cutlures, Erie, PA, USA; Pryce Haddix, Auburn University at Montgomery, Montgomery, AL, USA.

Mutagenesis and genetic manipulations

Transposon mutagenesis was performed as previously described [13] using Himar1 delivery plasmids pBT20 [14] and pSC189 [15]. Transposons were mapped by arbitrary PCR [16] or marker rescue [15]. After eight mutations were mapped to the *hexS* gene, primers that amplify the *hexS* ORF were used to screen transposon mutants with desired phenotypes. The primer sequences were GTTATTCTTC TTCGTCCACCAGGCTGG and ATGACAACTGCAAA TCGTCCGATACTTAATCTCG (all primer sequences are shown 5′ to 3′).

The *hexS* gene was mutated by allelic replacement as previously described using plasmid pMQ296 [17]. The pMQ296 plasmid was introduced into strains CMS2089 and CMS2097 by conjugation and was resolved using pMQ240, an I-*Sce*I delivery plasmid [18]. The *hexS* mutation was screened for by hyper-pigment phenotype, followed by PCR amplification and sequencing of the *hexS* gene to verify the *hexS*-Δ1 mutation. This mutation deletes one base pair of the *hexS* ORF causing a frameshift mutation and a null allele [17].

The *lrhA* gene was amplified from *E. coli* strain S17-1λ*pir* [19] using Phusion high-fidelity polymerase (New England Biolabs) and primers cgacggccagtgccaagcttgcatgcctgcaggtc-gacT-TACTCGATATCCCTTTCAATC and gtggaattgtgag-cggataacaatttcacacggaaacagATGATAAGTGCAAATCGTCC. The lower-case nucleotides target recombination and the upper-case letters direct amplification of the *lrhA* ORF, which was placed under control of the *E. coli lac* promoter on pMQ131 using yeast recombineering techniques [18, 20]. The resulting plasmid pMQ407 was introduced into *S. marcescens* by conjugation.

Mass spectrometry

Serratamolide analysis was performed as described previously [8, 21]. Bacteria were grown in LB medium for 20 h in 10×5 ml cultures per genotype and pooled. Cultures were centrifuged for 10 min at 10 000 ***g*** and 50 ml of the supernatant was extracted three times with an equal volume of ethyl acetate. The extract was dried over sodium sulphate and evaporated *in vacuo* and the residue was dissolved in methanol and analysed by HPLC-MS (Shimadzu LCMS-2020) equipped with a DIONEX Acclaim 120C18 column (3 µm particle size, 120 Å pore size, 2.1×150 mm dimensions).

A previously described [8], mobile-phase gradient was used along with a column flow rate of 0.2 ml min^{-1} at 40 °C. Serratamolide was monitored at *m*/*z*=515 with an ES-MS detector at positive mode, and purified serratamolide [21] was used as a positive control. The experiment was performed three times using independent bacterial cultures.

Gene expression analysis and electrophoretic mobility shift assays

β-Galactosidase assay: Bacteria with a plasmid-borne *flhDC-lacZ* transcriptional reporter, pMQ248, were grown in LB with kanamycin (100 μg ml^{-1}) overnight and then subcultured 1 : 100 into the same medium. After 20 h, samples were taken and the OD_{600} reading was determined with a spectrophotometer (Spectronic 200, Thermo Scientific). β-Galactosidase activity was determined as described by Griffith and Wolf [22].

Tdtomato assay: Bacteria with a plasmid-based *eepR* promoter fusion to *tdtomato*, pMQ412, were grown under the same experimental conditions described for the β-galactosidase assays noted above, and Tdtomato fluorescence was read as previously described [21] with a plate reader (Biotek, Synergy 2).

RNA preparation and quantitative reverse transcriptase PCR (qRT-PCR) were performed as previously described [8]. Primers for *eepR* (GGATTGGAAAACGTCAGCAT and CACGAAAAAGATGGCATCAC) and *hexS* (CGTTAAAGCGCAGGATCTTC and AAGAACCTTTGTTGCGGTTG) were designed to amplify DNA from the deletion alleles (all primers are listed as 5′ to 3′). Primer sequences for 16S and *prtS* analysis were noted in Brothers *et al.* [7]. Electrophoretic mobility shift assay (EMSA) reactions were performed with a commercial EMSA kit (Lightshift Chemiluminescent EMSA kit, Pierce) using previously described reagents (purified protein and promoter regions) and conditions [3, 8, 23]. The *hexS* promoter region was amplified using primers CCCGCGTTCTATAAGCACC and GCTCTAATCGCTGCATTTGTTG. The amplicon is 345 bp in length and includes 194 bp upstream of the *hexS* ORF that contains a predicted promoter determined using Softberry BPROM promoter prediction software. The *eepR*, *flhDC* and *prtS* promoter regions were as described previously [3, 8, 23]. Each EMSA experiment was performed three to six times with consistent results.

Statistical analysis

GraphPad Prism software was used for statistical analysis with significance set to $P<0.05$. Mann–Whitney *U*-tests were used for gene expression comparison and ANOVA with Tukey's post-test was used for other experiments as noted.

RESULTS

Suppressor analysis of the *eepR* mutant pigment defect

To gain insight into mechanisms by which EepR regulates pigment and co-regulated factors, suppressor analysis was performed. Random transposon mutations were introduced into a previously described Δ*eepR* mutant strain [8] that is pigmentless. Eighteen pigmented colonies were isolated from among 32 independent mutagenesis pools representing approximately 50 400 mutant colonies. A sample mutant screen plate is shown in Fig. 1(a). Pigmented strains were noted as red *eepR* suppressors (reep). A maximum of one pigmented colony was taken from each mutagenesis pool to eliminate sibling mutant colonies.

Transposon insertion sites were mapped in the majority of the reep strains. The mutations mapped to one of two locations: in the *hexS* ORF and upstream of the *eepR* ORF. This manuscript will describe the genetic interactions and transcriptional regulation of *eepR* and *hexS*. The mutations upstream of the *eepR* ORF will be described in a separate study.

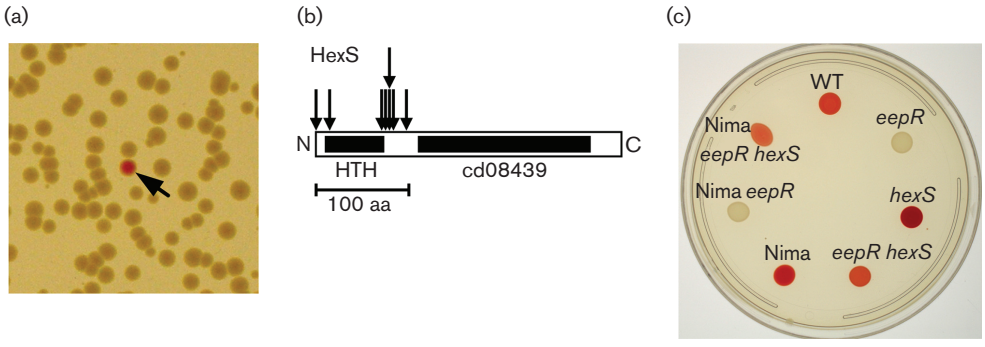

Fig. 1. Genetic screen for *eepR* suppressor mutations. Transposon mutations were introduced into the CMS2097 strain (Δ*eepR*) to find pigmented suppressor mutants. (a) A portion of one plate is shown with one red suppressor (reep) mutant (black arrow) visible among the pigmentless Δ*eepR* colonies. (b) Location of Δ*eepR* suppressor mutations (vertical arrows) in the *hexS* gene (horizontal bar). Of the 12 insertions, 8 are shown, the other 4 are in the *hexS* ORF but not mapped. (c) Prodigiosin pigmentation of strains grown on LB agar for 20 h at 30 °C. WT refers to parental strain PIC3611; *eepR*, to CMS2097; *hexS*, to CMS2922; *eepR hexS*, to CMS2204; Nima, to parental strain CMS1787; Nima *eepR*, to CMS2089; and Nima *eepR hexS*, to CMS2320.

Eight mutants had transposon insertions in the *hexS* gene at base pairs 1, 45, 210, 213, 214, 214, 265 and 292. Four more had mutations in the *hexS* ORF whose specific insertion sites were not mapped, as noted in Methods. The specifically mapped mutations clustered near the N-terminus of the HexS protein proximal to a helix–turn–helix domain, whereas none was isolated in a predicted cd08439 (substrate-binding domain) in the C-terminus. HexS is a LysR family transcription factor that directly and negatively regulates prodigiosin and serratamolide production by *S. marcescens* [3, 4, 17]. HexS is closely related to the LrhA protein of *E. coli* [24]. LrhA homologues, found in a variety of micro-organisms including *E. coli* (LrhA), *Erwinia* species (HexA and PecT), *Serratia* ATCC 39006 (PigU) and *Yersinia pseudotuberculosis* (RovM), are involved in regulation of secreted enzymes, motility and virulence [25–29].

The robust pigment phenotypes observed in this screen suggest a regulatory relationship between EepR and HexS in the coordination of secondary metabolite biosynthesis. We investigated whether this relationship went beyond prodigiosin, as previous studies demonstrate that EepR positively and HexS negatively regulates biosynthesis of the secondary metabolite serratamolide and proteases [4, 8].

Opposing control of serratamolide and serralysin biosynthesis by EepR and HexS

An *eepR hexS* double mutant strain was built incorporating the previously described *hexS*-Δ1 null mutation [17] by allelic exchange into the Δ*eepR* strain background. The double mutant strain was used for epistasis analysis to explore the relationship between EepR and HexS in coordinated regulation of secondary metabolites and secreted enzymes.

Introduction of the *hexS* mutation into the Δ*eepR* strain suppressed the pigment-defective phenotype of the *eepR* mutant (Fig. 1c). There were no obvious phenotypic differences observed between the reep mutants or between the reep mutants and the directed *eepR hexS* double mutant (CMS2204). Serratamolide is required for haemolysis and swarming phenotypes of many strains of *S. marcescens*, and PIC3611 harbouring the Δ*eepR* mutation (strain CMS2097) is unable to accomplish either phenotype due to a severe deficiency in serratamolide biosynthesis [8]. Both swarming ability and haemolysis were restored in the *eepR hexS* double mutant indicating that the double mutant synthesizes serratamolide (Fig. 2a, b). Swarming zone radii measured at 24 h for strains in the PIC3611 strain background were observed to be 3.0±1.7 mm for wild-type, 19.5±2.3 mm for Δ*hexS*, 0±0 for Δ*eepR* and 4.8±1.9 mm for Δ*eepR* Δ*hexS*. Importantly, these strains grew at similar rates indicating that the difference in motility is not due to altered growth by the mutant strains (Fig. 3a).

MS analysis was used to measure serratamolide production in the wild-type (PIC3611) and derived strains. Compared to the wild-type, increased levels of serratamolide were measured in the *hexS* and double mutant, and reduced serratamolide in the *eepR* mutant (Fig. 3b). The *eepR hexS*

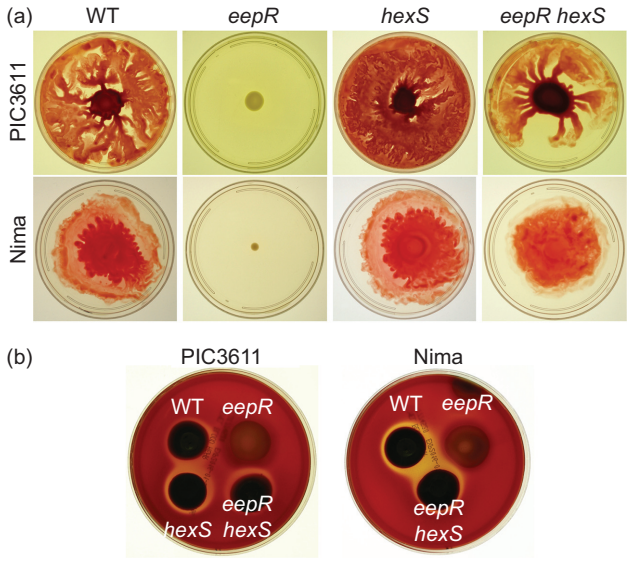

Fig. 2. Genetic evidence suggests coordinated swarming and haemolysis regulation by EepR and HexS. (a) Swarming motility after incubation for 20–48 h on LB medium with 0.6 % agar. (b) Haemolysis phenotype after 4 days of growth on tryptic soy agar +5 % sheep erythrocytes.

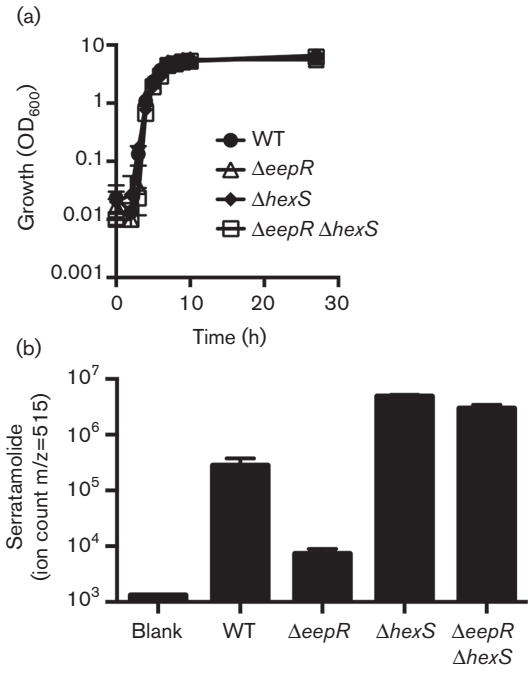

Fig. 3. Growth and MS analysis of serratamolide production by the *eepR hexS* double mutant. (a) Growth curve analysis of the wild-type strain PIC3611, Δ*eepR*, Δ*hexS* and Δ*eepR* Δ*hexS* in LB medium. (b) MS analysis of serratamolide biosynthesis of cultures grown for 24 h and normalized to OD_{600} 2.0. Means and standard deviations are shown, *n*=3 independent samples. PIC3611-derived strains were used.

double mutant produced serratamolide similarly to the *hexS* mutant, both significantly more than the Δ*eepR* mutant (P>0.001, ANOVA with Tukey's post-test). These data indicate epistasis of the *hexS* mutant phenotype over the *eepR* mutant phenotype for serratamolide biosynthesis.

Since EepR positively regulates secreted enzymes such as the cytotoxic metalloprotease serralysin (PrtS) [7] and HexS has been reported to regulate undetermined secreted protease(s) [4], we tested whether the overlapping regulons of HexS and EepR extend to secreted protease activity. Azocasein was used as a quantitative substrate to detect proteases in normalized stationary-phase culture filtrates. Similar to a study by Tanikawa *et al.* [4] who used a different strain background, the *hexS* mutant version of strain PIC3611 exhibited elevated protease activity. Unlike the *eepR* mutant, the *eepR hexS* double mutant produced protease activity similar to wild-type (Fig. 4a).

To ensure that this genetic interaction was not specific to strain PIC3611, we generated an *eepR hexS* double mutant variant of strain Nima [30]. Nima and PIC3611 are of different biotypes [31]. Pigment, serratamolide and protease activity were restored in a manner similar to strain PIC3611 and indicate that the relationship between HexS and EepR is not strain specific (Figs 1c, 2 and 4b).

Analysis of gene regulation by EepR and HexS

The suppression of several *eepR* mutant phenotypes by mutation of *hexS* led us to test whether HexS regulates *eepR* gene expression. The expression of *eepR* was increased 2.4-fold in the Δ*hexS* mutant compared to the wild-type (P<0.001, Fig. 5a). This trend of increased *eepR* expression was reversed when *hexS* was added to the chromosome *in cis* using pMQ294 as previously described [17] (P<0.01, ANOVA with Tukey's post-test; Fig. 5a). As a second way to measure the impact of *hexS* mutation on *eepR* expression, a P_{eepR}–*tdtomato* fusion was employed. Significant 4.8-fold and 3.1-fold increases in *eepR* expression were measured in the Δ*hexS* mutant relative to wild-type when measured at OD_{600} 1.2 and 3, respectively (P<0.05), data not shown. These results suggest that HexS inhibits *eepR* gene expression.

Experiments were carried out using qRT-PCR to test whether HexS mediates *hexS* expression. There was a non-significant twofold decrease in expression of the *hexS* gene in the *hexS* mutant strain compared to the wild-type strain when measured at OD_{600} 3 (0.029±0.012 for the wild-type and 0.014±0.002 for the *hexS* mutant, P=0.10).

A *hexS* mutant is known to produce more extracellular protease [4, 8], but the specific protease was not determined. EepR is known to regulate the serralysin protease, coded for by the *prtS* gene [7]. Given the potential overlap of the EepR and HexS regulons, we tested whether mutation of *hexS* changes *prtS* expression. A 37-fold increase in *prtS* expression (P=0.009) was measured by qRT-PCR from the Δ*hexS* mutant compared to the wild-type at OD_{600} 3 (Fig. 5b).

The role of EepR in transcriptional regulation of *hexS* and *eepR* was also tested. The *hexS* gene was elevated in expression 28-fold in the *eepR* mutant compared to the wild-type at OD_{600} 3 (Fig. 5c, P=0.0006). The *eepR* gene was also elevated in the *eepR* mutant, but only by 3.4-fold (P=0.016, Fig. 5d).

EMSA analysis was used to examine whether HexS and EepR directly regulated the genes tested for expression above. Formerly described maltose binding protein (MBP) protein fusions to EepR and HexS were used at previously optimized concentrations in promoter binding experiments; the MBP domain was used to affinity purify the fusion proteins and complementation analysis indicated that the fusion proteins retained functionality [3, 7, 8, 17]. Whereas MBP itself did not bind to the *eepR* promoter, the MBP–HexS fusion caused a gel shift of the biotinylated *eepR* promoter that could be inhibited by an excess of unlabelled *eepR* promoter DNA (Fig. 6a). Recombinant HexS did not bind to the *hexS* promoter region,

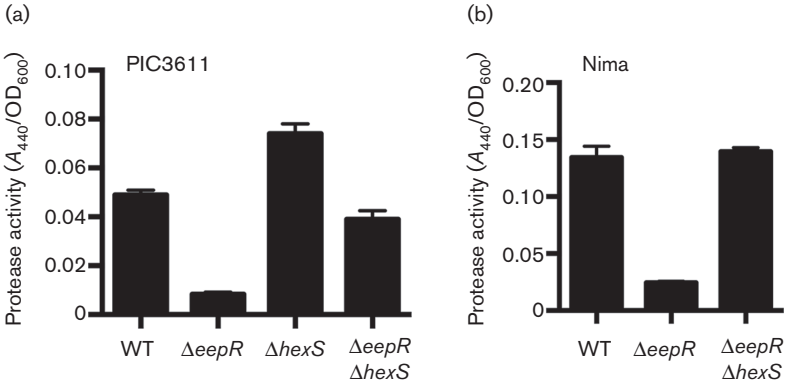

Fig. 4. Genetic evidence suggests coordinated protease regulation by EepR and HexS. Protease activity in supernatants from stationary-phase bacterial cultures normalized to OD_{600} 2.0. Azocasein was used as a colorimetric protease substrate. Means and standard deviations are shown, n=3 independent experiments, each with three biological replicates. (a) Protease activity from the PIC3611 background. (b) Protease activity from the Nima strain background.

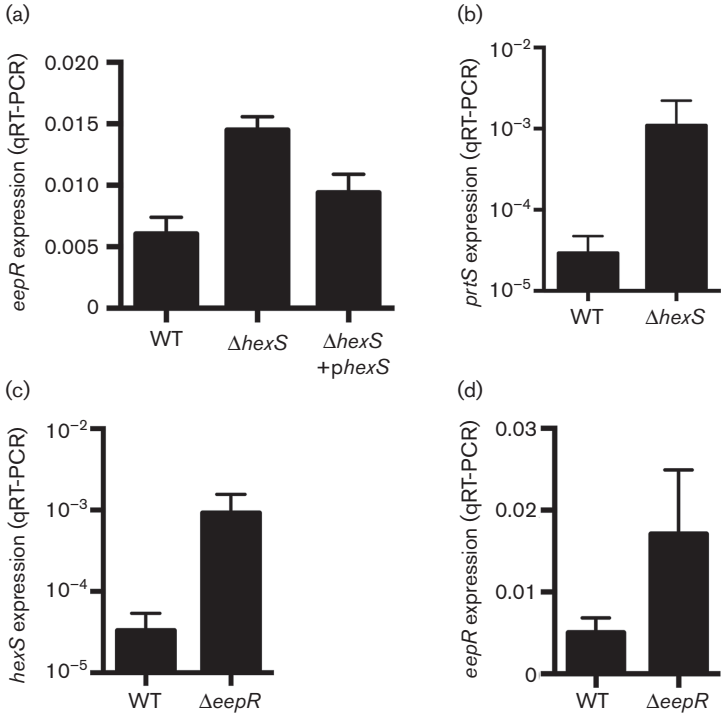

Fig. 5. Genes regulated by HexS and EepR. (a–d) qRT-PCR analysis of gene expression using RNA from strains grown to OD_{600} 3. (a, b) Analysis of *eepR* and *prtS* expression in WT compared to the Δ*hexS* mutant; (c, d) analysis of *hexS* and *eepR* expression in WT compared to the Δ*eepR* mutant. Gene expression was determined using PIC3611-derived strains. The p*hexS* plasmid refers to pMQ294. Means and standard deviations are shown. At least three independent replicates were used for each experiment.

suggesting that HexS does not directly regulate expression of the *hexS* gene (Fig. 6a). The absence of MBP–HexS binding to the *hexS* promoter also serves as a control for HexS binding specificity. Since HexS regulates secreted protease activity, we tested whether HexS bound to the *prtS* promoter, and we found evidence indicating that HexS could bind to the *prtS* promoter *in vitro* (Fig. 6a). Recombinant EepR bound to the *eepR* promoter, but not the *hexS* promoter region (Fig. 6b). The binding of EepR to *eepR* appears to be specific as the binding could be outcompeted with unlabelled *eepR* promoter sequence and recombinant EepR did not bind to the *hexS* promoter DNA. These data suggest that HexS directly regulates *eepR* and *prtS*, but not *hexS* expression, and that EepR directly regulates *eepR* expression but indirectly regulates *hexS* transcription.

Mulitcopy expressions of *hexS* and *lrhA* inhibit flagellum and prodigiosin biosynthesis and reveal a functional conservation

As noted above, HexS is similar to LrhA from *E. coli*. BLASTP analysis [32] indicates a 69 % amino acid sequence identity between HexS and LrhA. To test whether the HexS protein and LrhA are functionally related, we cloned the *lrhA* gene under control of the *E. coli* P_{lac} promoter and moved it into *S. marcescens*. Wild-type *S. marcescens* bearing *hexS* and *lrhA* on a medium-copy plasmid were both able to completely inhibit prodigiosin pigment production, whereas the vector control did not (Fig. 7a). This suggested remarkably conserved function as *E. coli* does not have the prodigiosin biosynthetic operon and yet multicopy expression of *lrhA* could impair pigment production similar to multicopy expression of *hexS*. Thus, former studies performed with LrhA in *E. coli* may give insight into other roles of HexS in *S. marcescens*. For example, *LrhA* regulates *flhDC* expression in *E. coli* [33–35]. FlhD and FlhC are the master regulators of flagellum biosynthesis and control biosynthesis of phospholipase and other metabolites in *Serratia* species [23, 36, 37]. Therefore, we tested whether HexS also regulates *flhDC*. A plasmid-borne *flhDC* promoter–*lacZ* fusion transcriptional reporter construct was introduced into the *hexS* mutant and isogenic wild-type strain. β-Galactosidase activity was >10-fold higher at OD_{600} 3 in the wild-type compared to the *hexS* mutant suggesting positive regulation of *flhDC* by HexS (Fig. 7b). EMSA analysis supports direct regulation of the *flhDC* promoter by HexS (Fig. 6a). However, this reduction in *flhDC* expression did not result in a corresponding loss in swimming motility under the tested conditions: the wild-type had a 46±7 mm swim diameter and the *hexS* mutant had a 44±7 mm swim zone in 24 h (P=0.58, Student's t-test).

DISCUSSION

The EepR regulator of *S. marcescens* is a global positive regulator of secreted enzymes and secondary metabolites and is necessary for wild-type levels of virulence in a

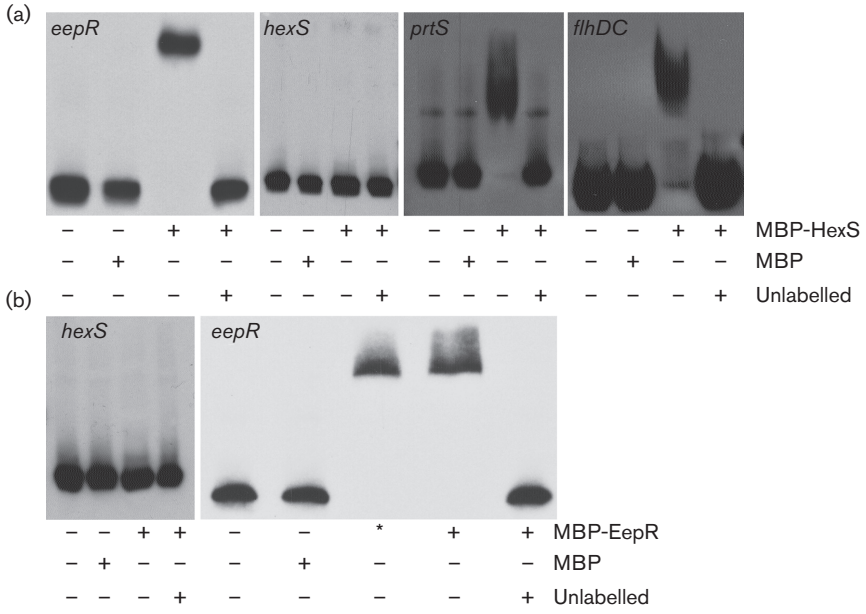

Fig. 6. EepR and HexS promoter binding analyses. EMSA analysis with biotinylated promoter DNA. Labelled promoter DNA was used in each reaction at 2 ng per reaction. Specific promoters are noted in the upper left-hand corner of each panel. MBP and MBP–HexS were used at 38 µM and 28 µM, respectively. MBP–EepR was used at 17.5 µM (*) or 35 µM (+); 500 ng of poly-dIdC was added per reaction to prevent non-specific protein–DNA interactions, and unlabelled promoters were used at 500 ng. –, indicates no addition of a particular reagent. (a) Representative EMSA using recombinant MBP or MBP–HexS. (b) Representative EMSA using recombinant MBP or MBP–EepR. Each EMSA experiment had a consistent result in at least three independent experiments.

rabbit keratitis model [7, 8]. The goal of this study was to use genetic suppressor analysis to find other regulatory factors that coordinate with EepR in control of the EepR transcriptional regulon. Suppressor mutations of the Δ*eepR* mutant pigment phenotype mapped to the *hexS* ORF.

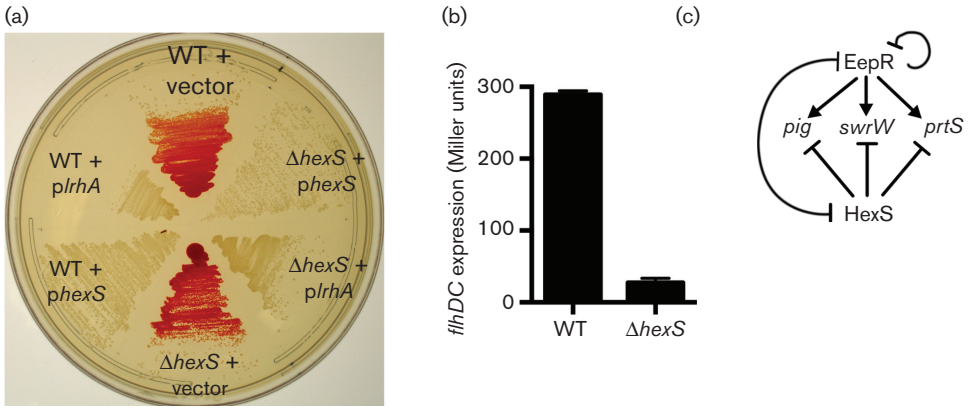

Fig. 7. Multicopy expressions of *lhrA* and *hexS* inhibit prodigiosin biosynthesis and *flhDC* expression in the *hexS* mutant. (a) Prodigiosin pigmentation of strains grown on LB agar with kanamycin for 20 h at 30 °C. Plasmids with the *hexS* or *lhrA* genes under control of the P_{lac} promoter inhibit pigmentation in both the wild-type and the Δ*hexS* mutant. Experiments were performed with the PIC3611 strain background and vector indicates pMQ131. (b) β-Galactosidase activity produced by WT and Δ*hexS* strains bearing a plasmid-borne *flhDC-lacZ* transcriptional reporter after growth in LB medium to OD_{600} 3. A representative experiment with three independent biological replicates is shown. Mean and standard deviation is shown. (c) Model, described in Discussion, for coordinated regulation of secondary metabolite biosynthetic genes (*pigA-N* and *swrW*) and the *prtS* protease gene by EepR and HexS. The line between EepR and HexS indicates that each inhibits transcription of the other.

The results presented here indicate that the *hexS* mutations not only suppressed the pigment defect of the *eepR* mutation but also were able to reverse other *eepR* mutant defects including loss of protease production, serratamolide biosynthesis and associated phenotypes, haemolysis and swarming motility. Notably, this study demonstrates that the elevated protease activity due to undetermined protease(s) generated by the *hexS* mutants is due, at least in part, to elevated production of PrtS. Importantly, suppression of the *eepR* mutant defects by mutation of *hexS* was consistent in two different strain backgrounds suggesting that EepR and HexS have a conserved relationship in control of the tested phenotypes.

Transcriptional and EMSA analyses suggest that EepR directly and negatively regulates expression of the *eepR* gene but indirectly regulates *hexS* expression in a strong negative manner. Evidence presented here supports the model that HexS negatively regulates expression of the *eepR* and *prtS*, but HexS did not bind to the *hexS* promoter and mutation of *hexS* did not cause a significant change in *hexS* transcript. The observation that, in the *hexS* mutant, *eepR* expression is elevated suggests that the derepression of the *eepR* promoter by *hexS* mutation is dominant compared to the negative regulation imparted by increased EepR. Together, these data suggest a model, Fig. 7 (c), in which EepR and HexS oppose one another in transcriptional control of secondary metabolites prodigiosin and serratamolide and of the cytotoxic protease serralysin (PrtS). In the absence of HexS, EepR is predicted to be made at higher levels, leading to increased pigmentation, serratamolide production and protease production. The opposite is also true where, in the absence of EepR, HexS is expected to be made at higher levels leading to a lack of secondary metabolite and protease production, as is seen in the *eepR* mutant. At this point, the signals stimulating EepR and HexS are unknown. EepR also appears to weakly inhibit the expression of the *eepR* gene, perhaps as a way to prevent the overproduction of energetically costly secondary metabolites, as overexpression of *eepR* has been shown to stimulate prodigiosin production [7, 8].

Lastly, the similarity between HexS and LrhA led to the surprising result that multicopy expression of *lrhA* was able to inhibit pigmentation in a similar manner to multicopy expression of *hexS*. This suggests that the two proteins have a highly conserved binding site and that genes controlled by LhrA in *E. coli* are likely to be controlled by HexS in *S. marcescens*. Unfortunately, the predicted LrhA binding site (AT-N_9-AT) [34] is common in the *S. marcescens* genome. Nevertheless, as an example of how we can take advantage of this similarity, we observed that the *flhDC* operon is regulated by HexS in *S. marescens* and a similar manner by LhrA in *E. coli*. However, the *flhDC* expression deficit in the *hexS* mutant did not result in a reduction in swimming motility through semisolid agar or in a reduction in swarming motility; this may be due to differences in liquid versus solid medium conditions. It is known that *S. marcescens* without flagella can swarm under certain conditions [38]. Importantly, these results indicate that the EepR-HexS regulon extends to FlhDC-regulated genes including flagella and phospholipase A [36], all of which may contribute to a bacterium's success in interspecies competition and pathogenesis.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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