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Serratia marcescens Cyclic AMP Receptor Protein Controls Transcription of EepR, a Novel Regulator of Antimicrobial Secondary Metabolites

Nicholas A. Stella,[a] Roni M. Lahr,[a]* Kimberly M. Brothers,[a] Eric J. Kalivoda,[a]* Kristin M. Hunt,[a]* Daniel H. Kwak,[b] Xinyu Liu,[b] Robert M. Q. Shanks[a]

Charles T. Campbell Laboratory of Ophthalmic Microbiology, Department of Ophthalmology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA[a]; Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, USA[b]

ABSTRACT

***Serratia marcescens* generates secondary metabolites and secreted enzymes, and it causes hospital infections and community-acquired ocular infections. Previous studies identified cyclic AMP (cAMP) receptor protein (CRP) as an indirect inhibitor of antimicrobial secondary metabolites. Here, we identified a putative two-component regulator that suppressed *crp* mutant phenotypes. Evidence supports that the putative response regulator *eepR* was directly transcriptionally inhibited by cAMP-CRP. EepR and the putative sensor kinase EepS were necessary for the biosynthesis of secondary metabolites, including prodigiosin- and serratamolide-dependent phenotypes, swarming motility, and hemolysis. Recombinant EepR bound to the prodigiosin and serratamolide promoters *in vitro*. Together, these data introduce a novel regulator of secondary metabolites that directly connects the broadly conserved metabolism regulator CRP with biosynthetic genes that may contribute to competition with other microbes.**

IMPORTANCE

This study identifies a new transcription factor that is directly controlled by a broadly conserved transcription factor, CRP. CRP is well studied in its role to help bacteria respond to the amount of nutrients in their environment. The new transcription factor EepR is essential for the bacterium *Serratia marcescens* to produce two biologically active compounds, prodigiosin and serratamolide. These two compounds are antimicrobial and may allow *S. marcescens* to compete for limited nutrients with other microorganisms. Results from this study tie together the CRP environmental nutrient sensor with a new regulator of antimicrobial compounds. Beyond microbial ecology, prodigiosin and serratamolide have therapeutic potential; therefore, understanding their regulation is important for both applied and basic science.

In order for organisms to survive and prosper, they must be able to sense their environment and effectively compete with other organisms. To respond to these environmental changes, bacteria have developed elaborate transcriptional regulatory systems that enable fine-tuning of factors that allow for their adaptation and proliferation. One of the most studied signaling systems involved in adaptation to the nutritive status of the environment is the cyclic AMP (cAMP)-associated catabolite repression system (1–4). The second messenger cAMP has been classified as an alarmone that induces positive regulation of alternative carbon transport systems in times of carbon/fuel deprivation (5). In addition to catabolite repression control, this system also can positively regulate flagellum production in unfavorable conditions (6) and activate attachment factors in nutrient-rich conditions (7).

Evidence suggests that cAMP-cAMP receptor protein (CRP) can directly bind to and promote expression of secondary metabolite genes involved in antibiotic production in *Streptomyces coelicolor* (2). A positive or negative role for cAMP has been suggested for control of antimicrobial production in other organisms, including fungi, although direct or indirect control of gene expression has not been determined (8–11). In general, cAMP-associated transcriptional circuits that regulate secondary metabolism are poorly understood.

The Gram-negative bacterium *S. marcescens* is known for its ability to produce numerous secondary metabolites (12–14). These include the surfactant serratamolide and the red pigment prodigiosin, which are broad-spectrum antibiotics that may aid the bacterium in competition, as well as having therapeutic potential for initiating apoptosis in cancer cells (15–17).

Mutation of genes involved in 3′-5′-cAMP production (*cyaA*) and the transcription factor that responds to cAMP (*crp*) confers robust phenotypes beyond catabolite repression, including increased prodigiosin production (18), elevated serratamolide pro-

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Address correspondence to Robert M. Q. Shanks, shanksrm@upmc.edu.

* Present address: Roni M. Lahr, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; Eric J. Kalivoda, Department of Emergency Medicine, St. John Hospital and Medical Center, Detroit, Michigan, USA; Kristin M. Hunt, School of Public Health, Boston University, Boston, Massachusetts, USA.

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duction (19), increased biofilm formation through increased type I pili production (20), enhanced extracellular protease production (21), and a loss of flagellum-based motility (22). CRP similarly regulates motility, adhesion, and secondary metabolite production by such diverse bacteria as *Salmonella enterica* serovar Typhimurium and *Streptomyces coelicolor* (1–4).

Whereas recombinant *S. marcescens* CRP bound directly to the promoter of *flhDC* to regulate flagellum production (18), interactions were not detected between recombinant CRP and promoters of the prodigiosin biosynthetic operon (*pigA-N*) or the *swrW* gene, required for serratamolide production (18, 21, 23). Based on these observations, we hypothesized an intermediate regulatory protein, regulated by cAMP-CRP, that in turn regulates expression of *pigA-N* and *swrW*. The purpose of this study was to identify this theoretical intermediate regulatory protein. Using a genetic approach, suppressor mutations of the hyperhemolysis and pigment phenotypes of the *crp* mutant strain were generated and mapped to an uncharacterized putative two-component transcriptional regulator locus. These genes, named *eepR* and *eepS*, were found to be a novel regulatory system for control of secondary metabolites by *S. marcescens*. The data further support that EepR is an intermediate transcriptional regulator between cAMP-CRP and secondary metabolite biosynthetic genes.

MATERIALS AND METHODS

Microbial strains, media, and growth. *S. marcescens* strains are listed in Table 1. Human keratitis isolate K949 was isolated at the Charles T. Campbell Laboratory Ophthalmic Microbiology Laboratory. Bacteria were grown with aeration in lysogeny broth (LB) medium (26) (0.5% yeast extract, 1% tryptone, 0.5% NaCl) with or without 1.5% agar, tryptic soy agar supplemented with 5% sheep erythrocytes (blood agar), or M9 minimal medium (27) supplemented with glucose (0.4%) and casein amino acids (0.06%). Swimming agar and swarming agar used in this study were LB medium with agar concentrations at 0.3% and 0.6%, respectively. *Escherichia coli* strains used were the EC100D *pir-116* (Epicentre), SM10 λ*pir*, and S17-1 λ*pir* strains (28). *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen) was grown with either yeast extract-peptone-dextrose (YPD) or synthetic complete (SC)-uracil medium (29). Antibiotics used in this study include gentamicin (10 μg ml^{-1}), kanamycin (100 μg ml^{-1}), and tetracycline (10 μg ml^{-1}).

Mutagenesis and plasmid construction. Transposons were introduced into *S. marcescens* by conjugation as previously described (20) using mariner-based transposon delivery plasmids pBT20 (30) and pSC189 (31). Tetracycline (10 μg ml^{-1}) was used to eliminate donor *E. coli* growth, and kanamycin (100 μg ml^{-1}) or gentamicin (10 μg ml^{-1}) was used to select for *S. marcescens* with transposon mutations. These were performed on blood agar plates to screen for pigment- and hemolysis-defective mutants as described below.

Cloning was performed using *in vivo* recombination (32) of PCR-generated amplicons or using T4 DNA ligase (New England BioLabs). PCR of amplicons used for cloning was performed using a high-fidelity polymerase, Phusion (New England BioLabs). Cloned genes were verified by diagnostic PCR and DNA sequencing (University of Pittsburgh Genomic and Proteomic Core). Plasmids are listed in Table S1 in the supplemental material. Directed mutagenesis was achieved by two-step allelic replacement or insertional mutagenesis as noted in the text and as previously described (21, 32). Mutations were verified using PCR primers outside the cloned region on the mutagenesis plasmid.

Allelic replacement of *eepR*, *eepS*, and *eepRS*. To generate the *eepS* deletion strain, we cloned *eepR*, including 458 bp upstream of *eepR* and the entire *eepS* open reading frame (ORF), here referred to as *eepRS*, in pMQ236 to generate pMQ289. Primers 1577 and 1578 were used to clone *eepRS*. Primers are listed in Table S2 in the supplemental material.

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

Strain	Description	Reference or source
CMS376	WT strain PIC3611	Presque Isle Cultures
CMS531	K949, clinical keratitis isolate	This study
CMS534	K949 with transposon upstream of *eepR*	This study
CMS592B	CMS376 with *pigB*::Tn (transposon insertion)	18
CMS613	CMS376 with *crp-1* null mutation	7
CMS635	CMS376 with *swrW*::Tn	23
CMS786	*crp-23* transposon null mutation	7
CMS794	CMS592B with *crp-1* null mutation	18
CMS795	CMS613 with transposon upstream of *eepR*	This study
CMS827	CMS376 with pMQ178 inserted in the *eepR* ORF	This study
CMS853	K904 clinical keratitis isolate	18
CMS1075	CMS376 with *crp-1 eepS*::Tn	This study
CMS1076	CMS1075 with restored *crp*	This study
CMS1464	CMS376 with *crp-23 eepS*::Tn	This study
CMS1687	Δ*crp-4* deletion null mutation	18
CMS1787	Nima pigmented environmental isolate	24
CMS2089	Nima with Δ*eepR*	This study
CMS2091	Nima with Δ*eepS*	This study
CMS2093	Nima with Δ*eepR* Δ*eepS*	This study
CMS2096	CMS376 Δ*pigP*	25
CMS2097	CMS376 Δ*eepR*	This study
CMS2157	CMS376 Δ*crp-4* Δ*eepR*	This study
CMS2395	CMS376 Δ*crp-4 eepS*::Tn	This study
CMS2701	CMS376 Δ*eepS*	This study
CMS2881	CMS376 Δ*crp-4 swrW*::Tn	15
CMS2904	K904 Δ*eepR*	This study
CMS2924	K904 with Δ*eepS*	This study
CMS2921	CMS2097 with Δ*eepR* replaced by wild-type *eepR*	This study
CMS2032	CMS1076 with *eepS*::Tn replaced with wild-type *eepS*	This study

To delete the *eepR* ORF, pMQ289 was digested with MluI and SalI, the ends were blunted with a multiple enzyme mixture (End-It kit; Epicentre), and the plasmid was recircularized using T4 DNA ligase. The resulting plasmid has an in-frame deletion of 67 out of 283 amino acids from E126-V192. The plasmid was named pMQ318z and used for allelic replacement. To make an insertion mutation in *eepR* at base pair 400 with respect to the translational start, a 359-bp internal fragment was amplified with primers 1234 and 1235 and cloned in pMQ118.

To mutate *eepS*, pMQ289 was digested with ApaLI, which cuts twice in *eepS*. The plasmid was recircularized using T4 DNA ligase, yielding the *eepS* deletion allele and plasmid pMQ308. This deletion is in frame and removes S337-H422 out of the total of 594 amino acids in EepS.

To generate the double *eepR eepS* mutation, pMQ289 was digested with AatII, which has sites in both *eepR* and *eepS*. The plasmid was recircularized using T4 DNA ligase, yielding the *eepRS* deletion allele. The resulting plasmid, pMQ291, has an in-frame deletion of the last 93 amino acids of *eepR* and an in-frame mutation of the last 79 amino acids of *eepS*.

To generate complementation vectors, the *eepR* open reading frame was cloned with primers that changed the start codon from ATG to TTG using primers 1222 and 2552 and placed the gene under the control of the *E. coli* P_{lac} promoter in plasmid pMQ132, yielding pMQ364, or in plasmid pMQ131, yielding pMQ432. A similar plasmid with a C-terminal polyhistidine (His_8) tag was generated with plasmid pMQ132, yielding pMQ369 using primers 2552 and 2698.

For purification of EepR, a maltose-binding protein (MBP)-EepR fusion construct was made with pMal-C2 (New England BioLabs). The *eepR* ORF was amplified with primers containing EcoRI and HindIII sites, and the restriction-digested amplicon was introduced into pMal-C2, which

also was digested with EcoRI and HindIII. Primers used to amplify *eepR* were MBP-R-R1 and MBP-R-H3. T4 DNA ligase was used to recombine the amplicon and vector to generate pMQ403.

To ensure that the MalE-EepR (MBP-EepR) fusion was functional, EepR and MalE ORFs were amplified from pMQ403 with primers 919 and 2948, and the amplicon was recombined into expression vector pMQ124. The resulting plasmid, pMQ438, has the MBP-EepR fusion under transcriptional control of the P_{BAD} promoter.

Prodigiosin production assays. Single colonies were inoculated in 5 ml of LB medium and incubated at 30°C for 16 to 18 h with aeration as noted above. Culture optical density was recorded, 1 ml of culture was transferred to a microcentrifuge tube, and the cells were pelleted. Prodigiosin was extracted from centrifuged cell pellets with 1 ml acidified ethanol (2 ml of 2 N HCl added to 98 ml of 95% ethanol), and pigment levels were measured by absorbance at 534 nm based on the method of Slater et al. (33), as previously described (18).

Transcriptional analysis. For β-galactosidase (β-gal) assays, cultures were grown overnight in LB medium with antibiotics at 30°C, subcultured (1:100) two times, and grown to an optical density at 600 nm (OD_{600}) of 0.1 in order to synchronize cultures in the early exponential growth phase. After growth to the desired optical density, culture aliquots were pelleted and washed with Z-buffer, and β-gal activity was determined (34). Lysates were prepared by sonication in Z-buffer and were clarified by centrifugation at 16,100 × *g* for 5 min. The supernatant protein concentration was determined by Bradford analysis, and the same amount of protein (0.2 mg) from each sample in a given experiment was added to microtiter plate wells. The volume was adjusted to 100 μl with Z-buffer. ONPG (*o*-nitrophenyl-β-D-galactopyranoside; 25 μl at 4 mg ml^{-1}) was added as a colorimetric substrate, and A_{410} readings were taken with a plate reader over a 30-min period (Biotek Synergy 2; Winooski, VT).

For *eepR* analysis, a transcriptional *lacZ* reporter construct was targeted into the chromosome of the wild-type and the *crp* mutant strains using a 263-bp region of DNA upstream of the *eepR* ORF containing the predicted CRP binding site in a *lacZ*-containing suicide vector (pMQ254). When this construct integrates, the promoter region is duplicated such that the *lacZ* gene becomes a reporter for *eepR* expression, and the native *eepR* gene comes under the control of the regulatory elements in the 263 bp of DNA upstream of the ORF, maintaining EepR, which may be necessary for *eepR* expression. For *pigA* and *swrW* promoter analysis, transcriptional *lacZ* fusions to internal fragments of the *pigB* and *swrW* genes (plasmids pMQ268 and pMQ223) were targeted to the chromosome by homologous recombination, verified by PCR, and used as previously described (18, 21, 23).

For quantitative PCR (qPCR) analysis, cultures for RNA extraction were grown at 30°C with aeration in 5 ml of LB broth following inoculation from a single colony. The overnight cultures were diluted to an OD_{600} of 0.1 in fresh LB medium, grown as described above, and harvested at the desired optical density. Bacterial aliquots were treated with RNAprotect bacterial reagent (Qiagen) by following the manufacturer's protocol and stored at −80°C for a maximum of 1 week. RNA was extracted (Qiagen RNeasy kit) using two rounds of DNase treatment (one Qiagen on-column DNase treatment and one Promega RQ1 DNase treatment) by following the manufacturer's protocols. RNA was concentrated using a Zymo Research RNA Clean & Concentrator-5 kit (R1015) according to the manufacturer's protocol. The RNA concentration was measured using a Thermo Scientific NanoDrop (model 2000) and normalized to 50 ng μl^{-1} using nuclease-free water. cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen) as specified by the manufacturer using 250 ng of RNA. The cDNA was diluted 1:5 in DNase-free water and tested for chromosomal DNA contamination by PCR using a thermal cycler (2720; Applied Biosystems) with oligonucleotide primers for the 16S rRNA gene (see Table S2 in the supplemental material) with amplification for 26 rounds, and any samples with a band on an agarose gel, indicating contamination, were discarded. Negative-control reactions without reverse transcriptase and without RNA were included and failed to produce an amplicon. Quantitative reverse transcription-PCR (qRT-PCR) was performed using Sybr green reagent (Applied Biosystems) according to the manufacturer's protocol using an Applied Biosystems Step One real-time PCR system with oligonucleotide primers listed in Table S2. The primers were 2638 and 2639 for the 16S rRNA gene, 1471 and 1472 for *eepR*, 2911 and 2912 for *pigA*, and 1786 and 2919 for *swrW*. qRT-PCR analysis was determined using the $\Delta\Delta C_T$ method (where C_T is threshold cycle).

Protein purification, electrophoretic mobility shift analysis (EMSA), and chromatin affinity precipitation (ChAP) assays. An MBP fusion to EepR (MBP-EepR) and MBP alone were generated for affinity purification of EepR using pMal-C2 (New England BioLabs) as previously described (25). His_8-CRP purification was described previously (18).

To perform EMSA, labeled DNA amplicons were made with a 5′-biotinylated oligonucleotide primer (Integrated DNA Technologies, Skokie, IL), gel purified, and verified by sequencing. A commercial EMSA kit was employed as specified by the manufacturer (LightShift chemiluminescent EMSA kit; Pierce, Rockford IL), using biotinylated target DNA (1 to 3 ng), purified His_8-CRP (≥50 ng), or MBP-EepR (≥50 ng) and poly(dI-dC) (500 ng), cAMP where indicated, and nonlabeled competitor DNA (20 to 600 ng) as specified, in a 20-μl reaction mixture. A 10-μl aliquot of the reaction mix was separated on a 5% PAGE, Tris-borate-EDTA (TBE) gel (Bio-Rad) with a running buffer containing 500 μM cAMP when His_8-CRP was used. EMSAs were repeated at least three times. Primers and amplified regions for the *oxyR*, *pigA*, and *swrW* promoter regions used in EMSAs have been described previously (25). Primers for the *eepR* promoter are 1346 and 1884, or a biotinylated version of primer 1884, which amplify a 263-bp region of DNA just upstream of the *eepR* start codon, using pMQ254 as a template. A 359-bp internal region of *eepR* was used to test the specificity of CRP binding to the *eepR* promoter interaction; this region was amplified using primers 1234 and 1235.

ChAP assays were performed as previously described (25), except using pMQ242 (His_8-CRP) and the pMQ124 vector as a negative control. Primer pairs for *flhDC*, *eepR*, and *oxyR* promoters were 1670 and 1671, 1667 and 1668, and 1432 and 1433, respectively. The experiment was repeated twice, yielding similar results.

Serratamolide measurement. Zones of biosurfactant around colonies on swarming agar plates were measured 18 to 20 h after inoculation of the bacteria onto the surface of the agar as previously reported (23, 25). Quantitative analysis of serratamolide from culture supernatants by high-performance liquid chromatography-mass spectrometry (HPLC-MS) was carried out as previously described (25). Bacterial cultures were grown in LB (10 5-ml cultures per genotype) for 20 h at 30°C. Bacteria were pelleted by centrifugation from pooled cultures, and the supernatant was extracted three times with equal volumes of ethyl acetate (30 ml). The ethyl acetate layers were dried over sodium sulfate and evaporated *in vacuo*. The dried residue was dissolved in methanol and analyzed by HPLC-MS (Shimadzu LCMS-2020) using a Dionex Acclaim 120 C_{18} column (3-μm particle size, 120-Å pore size; dimensions, 2.1 by 150 mm). A mobile-phase gradient, 40% acetonitrile (AcCN)–60% H_2O (0 min), 40% AcCN–60% H_2O (1 min), 90% AcCN–10% H_2O (15 min), 90% AcCN–10% H_2O (35 min), 40% AcCN–60% H_2O (40 min), and 40% AcCN–60% H_2O (45 min), was used for this analysis. The column flow rate was set to 0.2 ml min^{-1}, and the column oven temperature was set at 40°C. Serratamolide was monitored at $m/z = 515$ (for $[M+H]^+$) using an electrospray ionization-mass spectrometry (ESI-MS) detector in positive mode. Previously purified serratamolide (23) was used as a positive control.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software. One-way analysis of variance (ANOVA) with Tukey's posttest and the two-tailed Student's *t* tests were used with significance set at $P < 0.05$.

Nucleotide sequence accession number. The sequence of the *eepR* gene from strain CMS376 was deposited in GenBank under accession number JQ914138.

RESULTS

Identification of *eepR* and *eepS*. Previous studies demonstrated cAMP-CRP regulation of flagellum and secondary metabolite production by *S. marcescens* strain PIC3611 (18, 21, 23). Direct binding of cAMP-CRP to the promoter of the flagellar master regulator operon, *flhDC*, was observed, but a positive interaction between cAMP-CRP and promoters of the genes required for biosynthesis of the secondary metabolites prodigiosin (*pigA-N*) or serratamolide (*swrW*) was not (18). Because the regulation of flagella by cAMP-CRP involves intermediate regulators FlhD and FlhC (22, 35), we predicted an analogous intermediate regulator functions between CRP and *pigA-N* and *swrW*.

To find the predicted intermediate regulator downstream of cAMP-CRP, suppressor analysis was performed. Specifically, random mutations were introduced using a mariner transposon in a *crp* mutant background, and the mutant colonies were screened for suppression of the *crp* hyperprodigiosin phenotype. The mutants with reduced or eliminated prodigiosin then were screened for hyperhemolysis suppression phenotypes; an example is shown in Fig. 1A. A progidiosin-defective mutant with an insertion in the *pigB* pigment biosynthetic gene was included to demonstrate a strain defective in the pigment phenotype is not necessary for the hemolysis phenotypes (Fig. 1A). Hemolysis under the *crp pigB* colony is evident in Fig. 1A and shared by the *crp* mutant (not evident in Fig. 1A) but was absent from *crp eepR* and *crp eepS* mutants.

Mutations that suppressed both phenotypes of the *crp* mutant were mapped to one of two adjacent and convergently transcribed uncharacterized ORFs (Fig. 1B; also see Tables S3 and S4 in the supplemental material), corresponding to SMDB11_3958 and SMDB11_3959, respectively (ORF designations are based on the DB11 genome [36]), that are predicted to code for a two-component histidine kinase and a response regulator based on sequence.

Other transposon-based genetic screens that provided impetus to analyze these two ORFs included the following: (i) a mutation in SMDB11_3959 eliminated production of an antistaphylococcal compound produced by *S. marcescens* (15), (ii) multiple mutations of SMDB11_3958 eliminated secreted hemolysis activity by a clinical isolate, K904, and (iii) a mutation upstream of SMDB11_3959 eliminated protease secretion by a nonpigmented ocular clinical isolate, K949 (data not shown). Altogether, 12 independent mutations in SMDB11_3958 and SMDB11_3959 have been identified, and all of these mutant strains were found to be deficient in hemolysis (serratamolide) and, if the strain was pigmented, defective in prodigiosin production. Transposon insertion sites are listed in Tables S1 and S2 in the supplemental material. Based on these phenotypes and subsequent data, we are naming these genes exoenzyme and pigment response regulator and sensor kinase, i.e., *eepR* for the putative response regulator (SMDB11_3959) and *eepS* for the sensor histidine kinase (SMDB11_3958). The role of these genes in exoenzyme regulation will be described elsewhere. This study focuses on determining whether the *eepR* or *eepS* genes are CRP regulated and provides a primary characterization of this novel locus.

Analysis of EepR and EepS sequences. The *eepR* gene codes for a predicted 283-amino-acid response regulator transcription factor with an N-terminal helix-turn-helix domain of the BetR family and a C-terminal CheY receiver domain with a predicted phosphorylation site at D210 (Fig. 1C). The *eepR* gene from strain

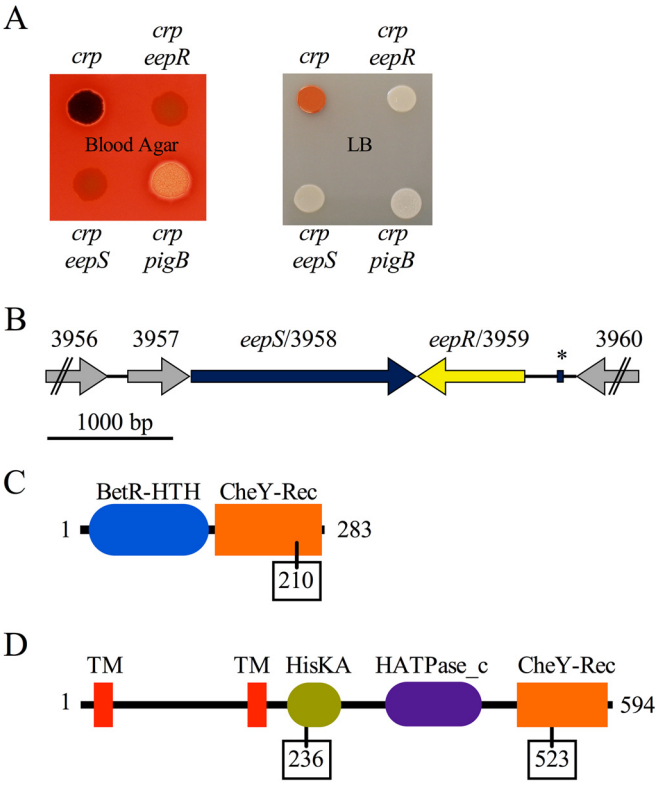

FIG 1 Hyperhemolysis phenotype of *crp* mutants and genetic analysis. (A) Photograph demonstrating suppression of the *crp* hemolysis (left) and pigment (right) phenotypes by mutation of *eepR* and *eepS*. The *eepR* and *eepS* colonies appear red because of the blood agar (left), but their severe lack of pigment phenotype is clear when grown on LB agar (right). The *crp pigB* mutant is included as a control and only has a defect in pigment production. The *crp* mutant is strain number CMS613, the *crp eepR* mutant is CMS795, the *crp eepS* mutant is CMS1075, and the *crp pigB* mutant is CMS794. (B) Map of the genetic context of *eepR* and *eepS*; ORF numbers are from the Db11 genome, and "SMDB11_" was removed from each ORF number to save space. The asterisk indicates a predicted CRP binding site. (C) Predicted protein domains and amino acid length of EepR. The amino acid location of the predicted phosphorylation site is boxed. (D) Predicted protein domains of EepS. The amino acid locations of predicted phosphorylation sites are boxed.

CMS376 was sequenced (GenBank accession number JQ914138) and shares 94.8% (DNA) identity with the corresponding DNA of a sequenced *S. marcescens* strain, Db11 (36); the resulting proteins from each strain are predicted to be 100% identical. Beyond other strains of *S. marcescens*, the predicted EepR protein is most similar to predicted response regulators in *S. plymuthica* (86% amino acid identity), other *Serratia* species (up to 79% amino acid identity), and various *Burkholderia* species (up to 50% amino acid identity).

The predicted EepS protein (Fig. 1D) is a 594-residue hybrid histidine kinase with two N-terminal transmembrane domains, a dimerization-photoreceptor domain (HisKA) with a predicted phosphorylation site at reside 236, a histidine kinase ATPase domain (HATPase_c), and a C-terminal CheY receiver domain with a predicted phosphorylation site at residue 523 (CheY-Rec). The organization of EepS suggests the existence of an intermediate phosphate carrier protein(s) between EepR and EepS (see Fig. S1 in the supplemental material). The AtsR protein from *B. cenocepacia* is 56.9% identical at the amino acid level and was found to mediate bacterial attachment to abiotic and biotic surfaces and

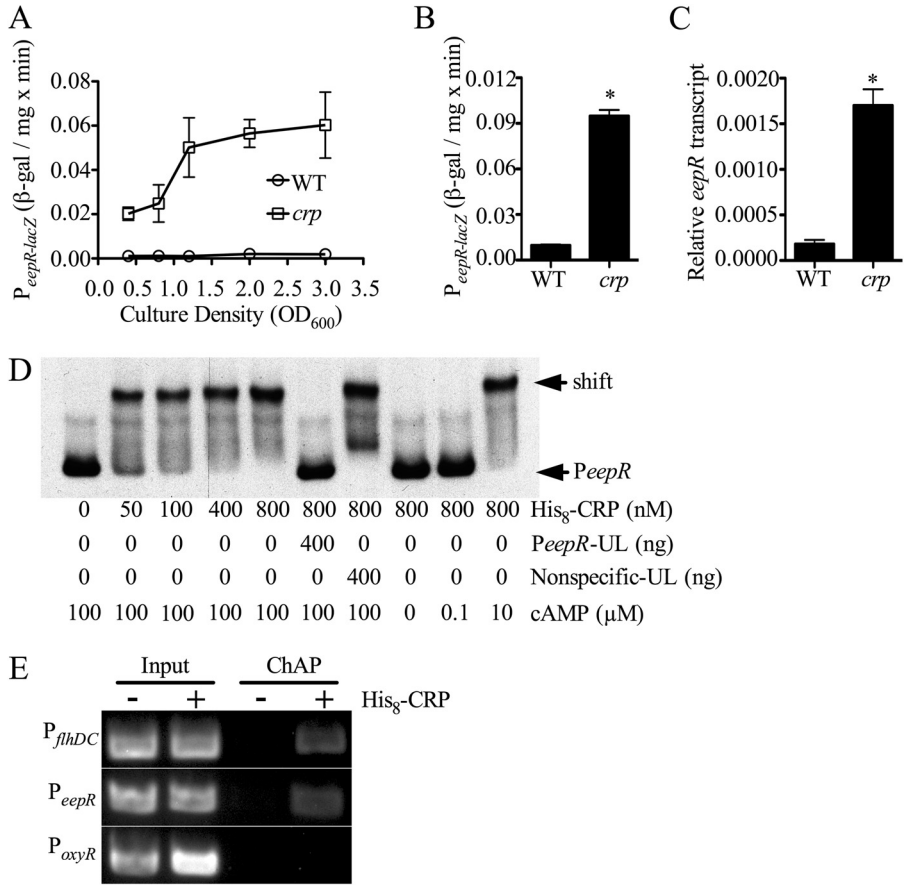

FIG 2 Direct regulation of *eepR* by cAMP-CRP. (A) Expression of the *eepR* promoter measured from a chromosomal *lacZ* reporter integrated at *eepR* in a WT and *crp* mutant background. The WT strain is CMS376, and the *crp* mutant is CMS786. (B) As described for panel A but at an OD_{600} of 4.0. (C) qPCR analysis of *eepR* expression from the WT and *crp* mutant measured at an OD_{600} of 1.5. The WT strain is CMS376, and the *crp* mutant is CMS1687. (D) EMSA of His_8-CRP interaction with the biotin-labeled *eepR* predicted promoter (P_{eepR}; 2 ng) *in vitro*. His_8-CRP produced a gel shift of labeled P_{eepR} that could be inhibited by an excess of unlabeled P_{eepR} (P_{eepR}-UL) but not by a nonspecific unlabeled amplicon (Nonspecific-UL), a 360-bp internal region of *eepR*. The gel shift required cAMP; whereas 0 and 0.1 μM did not support binding, 10 μM was sufficient. (E) Chromatin affinity purification of His_8-CRP suggests binding of the *eepR* promoter *in vivo*. PCR amplification of the *eepR* promoter was elevated from ChAP purification of CRP-bound DNA in the WT strain containing a plasmid expressing His_8-CRP (+CRP) compared to the WT strain with the empty vector (−CRP). The *flhDC* promoter was included as a positive control and the *oxyR* promoter as a negative control.

host-pathogen interactions in that opportunistic pathogen (37, 38).

The cAMP receptor protein directly regulates *eepR* expression. A predicted CRP-binding site was observed in the DNA sequence 241 to 257 bp upstream of the *eepR* ORF (TGAGACGAT GATCACA) (Fig. 1B, asterisk; also see Fig. S2 in the supplemental material), but none were noted upstream of the SMDB11_3957-*eepS* predicted operon. To test transcriptional regulation of *eepR* by CRP, a chromosomal *lacZ* fusion was used. In the WT strain, *eepR-lacZ* expression was low throughout growth but was highly elevated in the *crp* mutant strain (Fig. 2A). In separate experiments with cells grown for 20 h in LB medium with aeration (OD_{600} = ~4), β-galactosidase activity was 9.7-fold higher in the *crp* mutant than in the WT ($n = 9$) ($P < 0.01$) (Fig. 2B). qRT-PCR analysis of the native *eepR* gene agreed with the *lacZ* reporter data that there is a higher level of *eepR* transcript (9.5-fold) in the *crp* mutant than in the WT at an OD_{600} of 1.5 (Fig. 2C). As a third level of confirmation, the *tdtomato* fluorescent reporter gene was placed under the control of the *eepR* promoter (412 bp upstream of the *eepR* ORF) on a pBBR1-based plasmid. Higher levels of fluorescence were measured in the *crp* mutant (30,062 ± 1,186 relative fluorescence units [RFU]) cultures than in wild-type (11,812 ± 464 RFU) cultures grown overnight ($P < 0.01$ by Student's *t* test). Together, these data suggest a negative regulatory role for CRP on the *eepR* promoter.

EMSA was performed to test whether CRP protein binds to the *eepR* promoter *in vitro*. We observed that purified His_8-tagged recombinant CRP (His_8-CRP) bound to the *eepR* promoter in a dose-dependent and cAMP-dependent manner, and that CRP-*eepR* promoter interactions could be titrated with excess unlabeled *eepR* promoter DNA but not with unlabeled DNA internal to the *eepR* ORF, supporting that the interaction was specific (Fig. 2D). Furthermore, ChAP analysis was performed to test whether CRP binds to the *eepR* promoter *in vivo*. ChAP analysis was performed three times, and a semiquantitative analysis of PCR amplicon density indicates a 10.1- ± 1.8-fold increase in detection of the *eepR* promoter in the CRP pulldown samples (pMQ124+His_8-*crp*) compared to the level for the negative control (pMQ124) (Fig. 2E). A similar enrichment was found for the positive-control *flhDC* promoter but not the *oxyR* promoter,

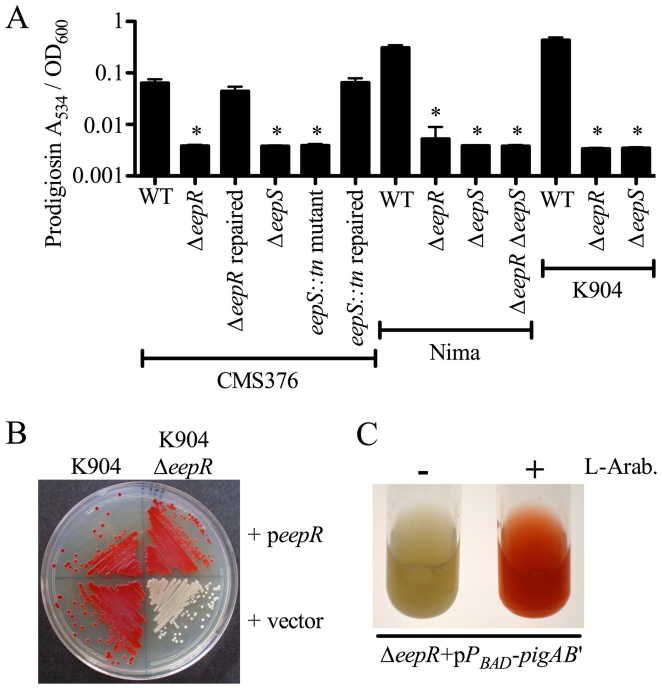

FIG 3 Growth and complementation analysis of *eepR* mutant pigment phenotypes. (A) Prodigiosin extracted and measured from stationary-phase bacteria. WT, CMS376; Δ*eepR* strain, CMS2097; Δ*eepR* repaired strain, CMS2921; Δ*eepS* strain, CMS2701; *eepS*::Tn strain, CMS1076; *eepS*::Tn repaired strain, CMS2032; Nima Δ*eepR* strain, CMS2089; Nima Δ*eepS* strain, CMS2091; Nima Δ*eepR* Δ*eepS* strain, CMS2093; K904 Δ*eepR* strain, CMS2094; K904 Δ*eepS* strain, CMS2924. Means and standard deviations are shown ($n = 8$). An asterisk indicates $P < 0.05$ by ANOVA with Tukey's posttest. (B) Complementation of the Δ*eepR* mutant phenotype in K904 and K904 Δ*eepR* strains using plasmid pMQ369 (p*eepR*) and a vector negative control (pMQ132). (C) Culture pigmentation in the Δ*eepR* strain (CMS2097) without (left) and with (right) L-arabinose (L-Arab.)-induced expression of the prodigiosin biosynthetic operon.

which serves as a negative control (Fig. 2E). Together, these data support the model that cAMP-CRP directly binds to the promoter of *eepR* and inhibits its expression (see Fig. S1A in the supplemental material).

EepR directly regulates prodigiosin biosynthesis. To further confirm that EepR and EepS play a role in prodigiosin production, we generated in-frame deletion mutations of *eepR* and *eepS* in WT strain CMS376. Both the *eepR* (CMS2097) and the *eepS* (CMS2701) deletion strains were defective in pigment production (Fig. 3A). Importantly, the *eepR* deletion mutation strains grown in M9 minimal medium were not defective in growth compared to the WT strain (see Fig. S1B in the supplemental material), indicating that the *eepR* mutant phenotypes are not due to reduced growth. Similar results were measured for growth of the CMS376 strain with an *eepS* transposon mutant, an *eepR* insertion mutant, and the parental strain grown in LB medium (see Fig. S1C). Furthermore, there were no detected differences between *eepR* and *eepS* mutants for the assays listed below; therefore, *eepR* mutant phenotypes alone generally will be shown for the sake of brevity.

Given the phenotypic variation caused by mutation of *crp* in *S. marcescens* and other *Enterobacteriaceae* (39, 40), we assessed whether *eepR* and *eepS* have a conserved role in other *S. marcescens* strains. The *eepR* and *eepS* genes were deleted from another laboratory strain, Nima, and from a contact lens-associated keratitis isolate, K904. Nima *eepR* and *eepS* and K904 *eepR* and *eepS* strains also lost pigment production (Fig. 3A and B). The K904 mutant differs from the other *eepR* mutants in that it eventually gained partial pigmentation when grown on plates for 2 days (data not shown). The K904 Δ*eepR* mutant was not growth defective (see Fig. S1B in the supplemental material). Strain Nima with deletion of both *eepR* and *eepS* was as defective in prodigiosin biosynthesis as either single mutant (Fig. 3A), suggesting a single pathway rather than EepR and EepS acting independently to promote pigmentation.

Complementation of the *eepR* mutant defect was performed to determine whether the deletion mutation rather than an unknown mutation elsewhere in the genome or a polar effect upon expression of adjacent genes was responsible for the observed phenotypes. In *trans* complementation of Δ*eepR* mutant phenotypes by expression of the wild-type *eepR* gene from the P_{lac} promoter on a pBBR1-based plasmid was performed (Fig. 3B; also see Fig. S1D in the supplemental material). In addition, allelic replacement of the *eepR* and *eepS* mutant alleles with the CMS376 wild-type (WT) genes restored pigment production (Fig. 3A and data not shown). This allelic replacement approach indicates that there is not a mutation elsewhere on the chromosome that caused the mutant phenotype, but it does not tell us whether the mutant phenotype was due to a polar effect. The similar phenotype conferred by multiple independent insertion mutations, in-frame deletion mutations in different strains, restoration of phenotypes by replacement of the mutant alleles with the wild-type genes, and expression of the wild-type *eepR* gene on a plasmid together support that mutation of the *eepR* gene rather than an unknown mutation or a polar effect confers the mutant phenotypes.

Consistent with EepR being a positive regulator of prodigiosin production, multicopy expression of the wild-type *eepR* gene from the P_{lac} promoter (pMQ369) in the WT strain (CMS376) increased prodigiosin production above the level of the vector-alone control (see Fig. S1D in the supplemental material). When prodigiosin was quantified from stationary-phase cultures there was almost twice as much isolated from the WT with multicopy *eepR* (A_{534}/OD_{600}, 0.23 ± 0.05) as there was for the WT with the vector control (0.12 ± 0.02; $n = 6$ per group; $P < 0.05$ by Student's *t* test).

The chromosomal prodigiosin biosynthetic operon was placed under the control of an arabinose-inducible promoter through integration of pMQ262 (15) in the Δ*eepR* strain (CMS2097) to test whether expression of *pigA-N* alone is sufficient to restore prodigiosin production. Red pigment production was restored when the strain was grown with the addition of the inducer L-arabinose but not when treated with glucose or water (Fig. 3C and data not shown). This observation supports that (i) induced expression of *pigA-N* is sufficient to restore pigmentation to an *eepR* mutant, (ii) the requirement of EepR in prodigiosin production is not downstream of *pigA-N* expression, and (iii) EepR has a regulatory rather than biochemical role in prodigiosin production. As a control for unintended effects of the L-arabinose sugar, we confirmed that the L-arabinose concentration used did not affect pigmentation of the *eepR* mutant without the pMQ262 plasmid (data not shown).

A chromosomal *lacZ* fusion and qPCR analysis both indicated that *eepR* is required for wild-type levels of expression from the prodigiosin biosynthetic operon *pigA-N* (Fig. 4A and B). Expression levels were more than 10-fold lower in the Δ*eepR* mutant than

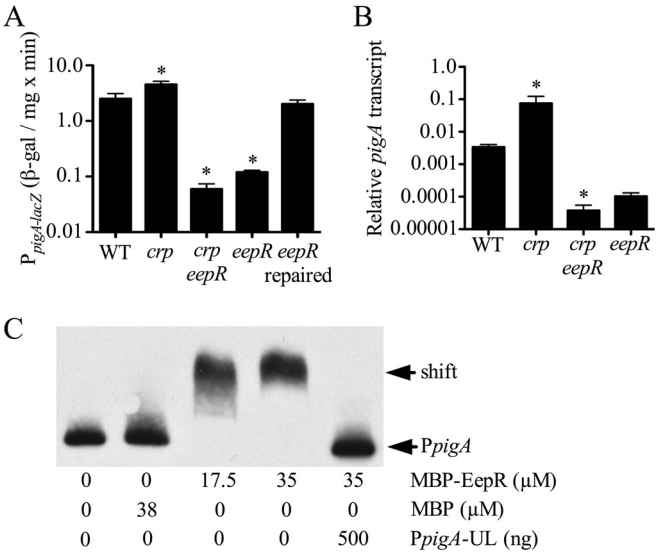

FIG 4 Regulation of *pigA* by EepR and epistasis analysis. (A) β-Galactosidase-based expression from the *pigA* promoter at an OD_{600} of 4. WT, CMS376; *crp* strain, CMS1687; *crp eepR* strain, CMS2157; *eepR* strain, CMS2097; *eepR* repaired strain, CMS2921. (B) qPCR analysis of the *pigA* promoter at an OD_{600} of 2. WT, CMS376; *crp* strain, CMS1687; *crp eepR* strain, CMS795; *eepR* strain, CMS2097. (C) EMSA analysis of MBP-EepR interaction with biotin-labeled *pigA* promoter (P_{pigA}; 2 ng) *in vitro*. MBP-EepR produced a gel shift of labeled P_{pigA} that could be inhibited by an excess of unlabeled P_{pigA} (P_{pigA}-UL). Recombinant MBP was not sufficient to produce a gel shift of P_{pigA}. An asterisk indicates significant difference from the WT by ANOVA with Tukey's posttest.

in the WT, with similar but slightly lower levels found in *crp eepR* double mutants compared to those of the *eepR* mutants (Fig. 4A and B). This lack of *pig* operon expression correlates with the lack of pigmentation in the *eepR* and *crp eepR* double mutant strains and are consistent with EepR and CRP functioning in a linear regulatory pathway (see Fig. S1A in the supplemental material).

A maltose-binding protein (MBP) fusion to EepR (MBP-EepR) was generated and found to be functional by complementation of the *eepR* mutant pigment phenotype (see Fig. S1D in the supplemental material). MBP-EepR bound to the *pigA-N* promoter *in vitro* in EMSAs, whereas purified MBP, by itself and at similar concentrations, was unable to bind to the *pigA* promoter (Fig. 4C). Furthermore, the MBP-EepR interaction with the labeled *pigA-N* promoter could be outcompeted using an excess of unlabeled *pigA-N* promoter DNA (Fig. 4C). We observed that MBP-EepR did not bind to other candidate promoters, such as the *gdhS* glucose-dehydrogenase and *pigP* transcription factor genes, lending additional evidence to the idea that the MBP-EepR binding to the *pigA-N* promoter is specific (see Fig. S3 in the supplemental material). Together, these data support that EepR directly and positively regulates *pigA-N*.

EepR/S is required for serratamolide production, hemolysis, and swarming motility. Mutation of *crp* in CMS376 results in higher levels of hemolysis than that of the isogenic parental strain due to increased production of the biosurfactant serratamolide (23), also known as serrawettin W1 (41). Since EepR/S appears to function with CRP in a regulatory pathway that controls prodigiosin production and EepR and EepS are required for the hemolysis phenotype of *crp* mutants (Fig. 1B), we tested whether EepR played a role in serratamolide production. Mutation of *eepR* and *eepS* in other isolates, such as K904 and Nima, eliminated hemolysis as measured from clearing zones on blood agar plates (Fig. 5A and data not shown).

Serratamolide also is required for swarming motility in many strains of *S. marcescens* (25, 42). We observed that *eepR* and *eepS* mutants were defective in swarming in the wild-type (CMS376) and K904 strain backgrounds (Fig. 5A and B). The *eepR* mutant swarming defect was complemented when the wild-type *eepR* gene was used to replace the deletion allele on the chromosome (Fig. 5B). Whereas swarming motility was defective, swimming motility was equivalent to that of the wild type (Fig. 5A). This result was consistent with the swarming defect resulting from reduced surfactant production rather than a deficiency in functional flagella. Expression of the serratamolide biosynthetic gene, *swrW*, from a multicopy plasmid restored swarming motility and hemolysis to tested *eepR* and *eepS* mutants (Fig. 5C and data not shown), suggesting that a loss of *swrW* expression was the mechanism underlying the *eepR* hemolysis and swarming defects.

Similar to *swrW* mutants, strains defective in *eepR* and *eepS* exhibited no zones of surfactant around colonies, whereas WT colonies produced surfactant zones extending about 4 mm beyond the colony edge by 24 h (Fig. 5D). The *crp* mutant was previously shown to generate zones larger than those of the WT (23). Here, we observed that the *crp* mutant produced an average zone of 7 mm, whereas the *crp eepR* and *crp eepS* double mutants, as well as the *crp swrW* strain, produced no zone of surfactant (Fig. 5D).

HPLC-MS analysis verified that serratamolide is reduced in spent supernatants produced by *eepR* mutants (CMS2097) grown to saturation (Fig. 5E). Unlike the WT, which exhibits a large serratamolide peak, the *eepR* mutant extracts were almost completely devoid of detectable serratamolide, much like the negative-control *swrW* mutant (CMS635) that is unable to generate serratamolide (Fig. 5E). Supernatants from the K904 strain and its isogenic *eepR* mutant were similarly defective in serratamolide when assessed by HPLC-MS (data not shown). Interestingly, the *eepR* mutant was more defective than a *pigP* deletion mutant (Fig. 5E). PigP is a previously described positive regulator of serratamolide production in *S. marcescens* (25).

To test the prediction that EepR regulates expression of the *swrW* gene, a chromosomal *lacZ* reporter and qPCR were used to measure *swrW* expression from cells grown for 18 h at 30°C. Expression was significantly higher when measured from the wild-type strain compared to that of the Δ*eepR* mutant and Δ*eepS* mutant in the CMS376 strain background (Fig. 6A). Results from qPCR indicate the WT had 21- ± 6-fold higher *swrW* expression than the Δ*eepR* mutant in stationary-phase cells (OD_{600} of 3.0) (Fig. 6B). EMSA was used to assess whether recombinant EepR (MBP-EepR) was able to bind directly to the *swrW* promoter *in vitro*, unlike the MBP negative control (Fig. 6C). As with the *pigA-N* promoter described above, an MBP-EepR–*swrW* promoter interaction was observed, suggesting that EepR positively and directly regulates the serratamolide biosynthetic gene.

DISCUSSION

The previously undescribed *eepR* and *eepS* genes were identified in a number of genetic screens in different strain backgrounds that were focused on determining regulators of secondary metabolites. Evidence from this study suggests that EepR directly and positively regulates the *pigA* and *swrW* promoters, making EepR the

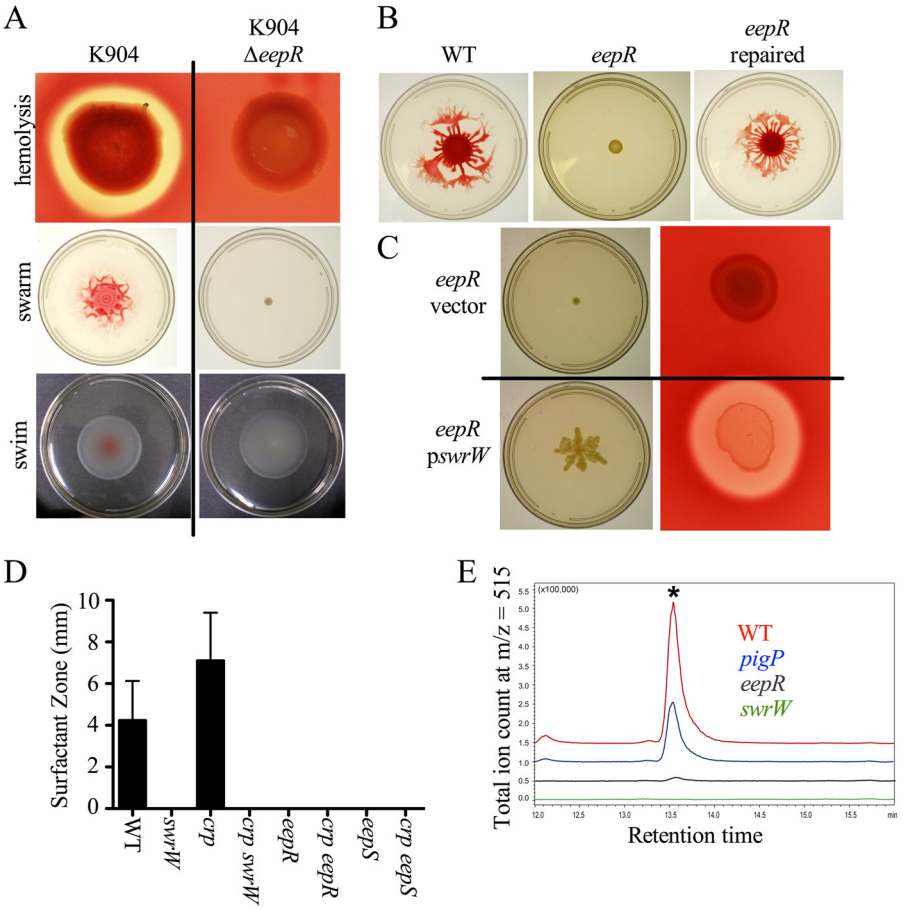

FIG 5 EepR is necessary for serratamolide and serratamolide-dependent phenotypes. (A) Hemolysis and swarming are EepR dependent in strain K904, but swimming is not. K904 Δ*eepR* mutant, CMS2904. (B) Swarming motility is defective in the Δ*eepR* (CMS2097) mutant and could be restored when the chromosomal *eepR* deletion allele was replaced by the wild-type *eepR* gene (CMS2921). (C) Hemolysis and swarming phenotypes of the Δ*eepR* mutant (CMS2097) can be rescued by induced expression of *swrW* on a plasmid (p*swrW*/pMQ367) but not by the vector control (pMQ125). (D) Means and standard deviations from surfactant radii around colonies on a swarming agar plate ($n \geq 5$ independent isolates). WT, CMS376; *swrW* strain, CMS635; *crp* strain, CMS1687; *crp swrW* strain, CMS2281; *eepR* strain, CMS2097; *crp eepR* strain, CMS2701; *crp eepS* strain, CMS2395. (E) HPLC-MS analysis of serratamolide levels in supernatants from stationary-phase cultures. An asterisk indicates the serratamolide peak. WT, CMS376; *pigP* strain, CMS2096; *eepR* strain, CMS2097; *swrW* strain, CMS635).

only described direct and positive regulator of *swrW* transcription. Furthermore, the data presented here support the model that EepR is the hypothetical intermediate regulator in the cAMP-CRP-mediated pathway postulated at the onset of the project (see Fig. S1A in the supplemental material). To our knowledge, this is the first example of a CRP family protein regulating production of antimicrobial secondary metabolites through an intermediate regulator. This multiregulator model may be a common theme. Consistent with this, of the eight secondary metabolite gene clusters regulated by CRP in *Streptomyces coelicolor*, only six were directly regulated (2). This outcome ties a major metabolic regulator, the catabolite repression system (cAMP-CRP), to the regulation of factors that likely aid in competition. Serratamolide and prodigiosin both are reported to kill or inhibit a wide range of microbes in order to limit competition for nutrients. In addition, serratamolide and prodigiosin can be toxic to eukaryotic cells, which can provide an additional source of factors, such as iron, important for bacterial growth. Since cAMP is reduced under high nutrient conditions, it is expected that these EepR levels would increase, leading to greater production of competition factors in a favorable niche.

Another potential benefit for secondary metabolite regulation to be tied to a central metabolic regulator, cAMP-CRP, is suggested by Haddix et al.; prodigiosin was shown to have a role in energy spilling that may protect cells at high culture density (43). Furthermore, recent work supports that prodigiosin has a positive effect on cell yield (P. Haddix, personal communication). It is possible that prodigiosin, like other bacterial pigments, protects the bacterium from metabolism-derived oxidative stress fitting, with a model where low cAMP levels derepressing *eepR* expression should lead to increased prodigiosin production.

cAMP-CRP directly and positively regulates transcription of the flagellum master regulator in *S. marcescens*, *flhDC* (22), and negatively regulates biofilm formation through regulation of type I pili (20). Together these data lead to a simplified model in which cAMP levels are elevated under low-nutrient conditions, shutting off biofilm formation and *eepR* expression and turning on FlhDC, so that the bacterium can seek a more favorable environment.

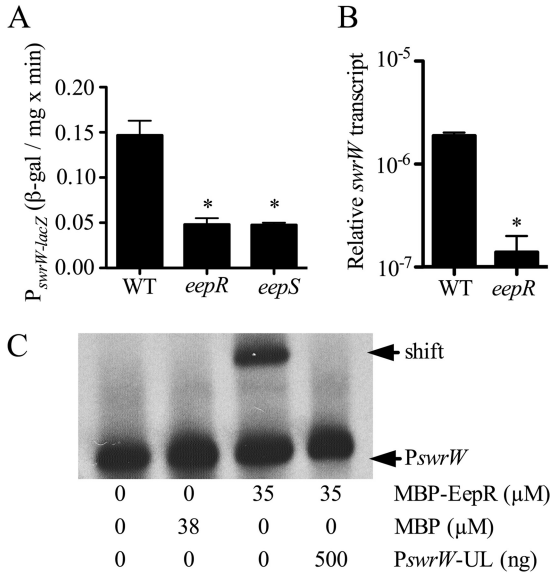

FIG 6 Positive regulation of serratamolide biosynthetic gene, *swrW*, by EepR. (A) β-Galactosidase analysis of *swrW* expression from cultures at an OD_{600} of 4. Means and standard deviations are shown. An asterisk indicates significant difference from results for the WT by ANOVA with Tukey's posttest. WT, CMS376; *eepR* strain, CMS2097; *eepS* strain, CMS1076. (B) Quantitative RT-PCR analysis of *swrW* expression from cultures at an OD_{600} of 3. Means and standard deviations are shown. An asterisk indicates significant differences by Student's *t* test. WT, CMS376; *eepR* strain, CMS2097. (C) EMSA analysis of MBP-EepR interaction with biotin-labeled *swrW* promoter (P_{swrW}; 2 ng) *in vitro*. MBP-EepR produced a gel shift of labeled P_{swrW} that could be inhibited by an excess of unlabeled P_{swrW} (P_{swrW}-UL). Recombinant MBP was not sufficient to produce a gel shift of P_{swrW}.

Under ideal conditions, such as a glucose-rich environment, cAMP levels will be low, deactivating flagellum production and turning on biofilm formation and EepR-regulated nutrient acquisition enzymes and competition factors.

The sequences and phenotypes of mutant strains suggest that EepS and EepR are a histidine sensor kinase and response regulator pair; however, further biochemical and/or directed mutagenesis analysis will be required to formally demonstrate that EepS and EepR together form a two-component regulatory system. Moreover, the amino acid sequence of EepS suggests that, rather than being part of a simple two-component system, it will require an intermediate phosphocarrier protein to phosphorylate EepR. Although we demonstrated here that cAMP-CRP negatively regulates *eepR* transcription, the impact of EepS on EepR activity is less clear. The signal for most histidine kinases is unknown and notoriously difficult to determine. Nevertheless, as a sensor kinase, EepS is predicted to regulate EepR activity in response to some additional signal, for example, through an unknown intermediate phosphocarrier protein (see Fig. S1 in the supplemental material). This may fine-tune or serve as an override switch of cAMP-CRP regulation of EepR, when, for example, EepR-regulated genes are necessary or deleterious. Interestingly, recombinant EepR bound to the *pigA* and *swrW* promoters *in vitro* in the absence of a phosphodonor such as carbamoyl phosphate. One possible model is that EepR is active in a nonphosphorylated form; therefore, EepS may promote secondary metabolite production by removing phosphate from EepR rather than acting as a kinase.

We recently reported that the *S. marcescens* PigP transcription factor positively regulates *pigA-N* directly and *swrW* indirectly. PigP was necessary for the hyperprodigiosin and hyperserratamolide phenotypes of *crp* mutants, and *pigP* expression was regulated by cAMP-CRP. However, CRP did not directly regulate transcription of *pigP*, suggesting that PigP, while involved in the same pathway, was not the missing regulator directly controlled by CRP. Extensive work with another *Serratia* species, ATCC 39006, demonstrated that secondary metabolism biosynthetic genes are regulated by quorum-sensing, cyclic-di-GMP, gluconate, phosphate, temperature, and other signals (17, 44–50). The existence of multiple regulators involved in controlling these factors (secondary metabolites, secreted enzymes, flagella, and adhesins) underscores the complexity of the regulation, the large number of external stimuli that must be coordinated, and the energy investment involved in making these factors.

In conclusion, this study introduces two genes predicted to code for a two-component transcriptional regulatory system composed of EepR as a putative response regulator and EepS as a putative sensor kinase. These two genes have an important impact on a variety of processes by *S. marcescens*, including motility, hemolysis, and production of antimicrobial compounds that likely play a role in bacterium-bacterium interactions and successful colonization of environmental niches.

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