NIH Public Access
Author Manuscript
Res Microbiol. Author manuscript; available in PMC 2011 March 1.

Published in final edited form as:
Res Microbiol. 2010 March ; 161(2): 158–167. doi:10.1016/j.resmic.2009.12.004.

Cyclic AMP negatively regulates prodigiosin production by *Serratia marcescens*

Eric J. Kalivoda[1,2], **Nicholas A. Stella**[1], **Marissa A. Aston**, **James E. Fender**, **Paul P. Thompson**, **Regis P. Kowalski**, and **Robert M. Q. Shanks**[*]
Charles T. Campbell Laboratory of Ophthalmic Microbiology, Department of Ophthalmology, University of Pittsburgh Eye Center, Pittsburgh, PA 15213, USA

Abstract

Many *Serratia marcescens* strains produce the red pigment prodigiosin, which has antimicrobial and anti-tumor properties. Previous reports suggest that cyclic AMP (cAMP) is a positive regulator of prodigiosin production. Supporting this model, the addition of glucose to growth medium inhibited pigment production in rich and minimal media. Unexpectedly, we observed highly elevated levels of prodigiosin production in isogenic strains with mutations in genes involved in cAMP production (*cyaA* and *crr*) and in cAMP-dependent transcriptional signaling (*crp*). Multicopy expression of the *Escherichia coli* camp phosphodiesterase gene, *cpdA*, also conferred a striking increase in prodigiosin production. Exogenous cAMP decreased both pigment production and *pigA-lacZ* transcription in the wild-type (WT) strain, and *pigA-lacZ* transcription was significantly increased in a *crp* mutant relative to WT. Suppressor and epistasis analysis indicate that the hyperpigment phenotype was dependent upon pigment biosynthetic genes (*pigA*, *pigB*, *pigC*, *pigD* and *pigM*). These experiments establish cAMP as a negative regulator of prodigiosin production in *S. marcescens*.

Keywords

cAMP; Catabolite repression; Pigment; Prodigiosin

1. Introduction

S. marcescens is a Gram-negative bacterium that is well known for its red pigment prodigiosin. This pigment has a tri-pyrrole structure and may have a role in competition for environmental niches, as it has antimicrobial activity, reviewed by [11,36]. Reports indicate that prodigiosin could function as an anti-cancer compound [24,27]. Prodigiosin has also been correlated with hydrophobicity-mediated bacterial adhesion and aerosol-based dispersion [3,28].

The cyclic nucleotide 3′,5′-cyclic AMP (cAMP) is a signaling molecule in prokaryotes that has a global impact upon gene expression through binding with the transcription factor, cAMP-receptor protein (CRP), which can positively and negatively regulate expression of target genes

© 2009 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.

* Corresponding author: shanksrm@upmc.edu; phone: 412-647-3537 fax: 412-647-5880.
[1]These authors contributed equally to this manuscript
[2]Present address University of Vermont, College of Medicine

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

[2,6,19]. Cellular levels of cAMP are positively controlled by adenylate cyclase activity, which is inhibited by glucose [2]. Bacteria import glucose using the phosphoenolpyruvate phosphotransferase system (PTS). One of the PTS components, coded for by the *crr* gene, is enzyme IIA^{Glc}. When glucose is limiting, enzyme IIA^{Glc} becomes phosphorylated and activates adenylate cyclase (CyaA) to generate cAMP. Conversely, when glucose is abundant, enzyme IIA^{Glc} does not become phoshphorylated or activate CyaA, leading to lower intracellular levels of cAMP [2,21]. Levels of cellular cAMP can be further modulated by the action of a cAMP-phosphodiesterase, which hydrolyzes cAMP, yielding 5′-AMP [17].

Previous studies suggest a positive role for cAMP in regulation of prodigiosin production. One report showed that exogenous glucose inhibited pigment [4]. Another report, by Winkler and colleagues, described strains with unmapped mutations that lacked cAMP-phosphodiesterase and adenylate cyclase activity, and briefly mentioned that these strains were colorless and that pigment could be restored with exogenous cAMP, though no data was shown [40]. Similarly, a transposon mutation in a predicted CyaB-like class IV adenylate cyclase gene was reported in another *Serratia* species, and was reported to confer a ~30% decrease in prodigiosin production, but its potential role as an adenylate cyclase was not addressed [9]. Together, these led to the model that cAMP was a positive regulator of pigment production.

Genetic studies in *Serratia* sp. ATCC 39006 have shown that pigment production has a large number of regulators including PigP, PigQ, PigR, PigS, PigV, PigX, and Rap [9]. Other reported prodigiosin modulating factors include different carbon sources [8,12,14], phosphate [32], quorum sensing [5,9,25,35], temperature [1,37], ATP [14], and most likely cyclic-di-GMP [10].

Recently, *S. marcescens* cAMP-associated genes have been identified and mutated [18]. Strains with mutation of *cyaA* and *crr* exhibited a severe reduction in intracellular camp levels, and strains with *crp* mutations were unresponsive to exogenous cAMP [18,33]. One unreported phenotype of these mutations was a striking increase in red pigment production, raising the question of whether cAMP positively regulates prodigiosin production as previously proposed. Here we provide evidence that cAMP negatively regulates prodigiosin production.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All strains and plasmids are listed in Table 1. All bacteria were grown in LB (0.5% yeast extract, 1% tryptone, 0.5% NaCl). LB broth was supplemented with adenosine 3′, 5′-cyclic monophosphate (A9501, Sigma-Aldrich, Inc.) or glucose where noted, with the supplement dissolved in LB medium, which was subsequently filter-sterilized. M63 medium supplemented with casein amino acids (0.08% w/v), glucose (0.4-4.0% w/v) and agar (1.5% w/v) was used for one pigment experiment. All experiments were performed at 30°C. The antibiotics used were gentamicin (10 μg/ml), kanamycin (100 μg/ml), and tetracycline (10 μg/ml). The strain CHASM (compost heap- acquired *S. marcescens*) was isolated from a compost pile, as a red-pigmented colony on *Pseudomonas* isolation agar (Biomérieux, Lyon, France), which also selects for *S. marcescens*. This red-pigmented strain was observed to be a Gram-negative rod that was cytochrome-oxidase-negative (BD diagnostics systems, Franklin Lakes NJ). An API 20 E (Biomérieux, Lyon, France) test strip was inoculated with a bacterial suspension (as per manufacturer's instructions) and the resulting profile compared to the API 20 E analytical profile index was consistent with the identified red bacteria being *S. marcescens*. K904 is a pigmented ocular clinical isolate of *S. marcescens* from the University of Pittsburgh Eye Center.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2.2. Mutagenesis and plasmid construction

All plasmids were introduced into *S. marcescens* by conjugation using either strain SM10 λpir or S17-1 λpir. Briefly, 0.5 ml of overnight cultures from donor and acceptor strains were concentrated by centrifugation, heat-shocked at 42°C for 15 min and plated in small pools on LB agar. After 10-24 h, the entire pool was streaked to single colonies on LB agar with tetracycline to select against donor *Escherichia coli* and either kanamycin or gentamicin.

Oligonucleotide primer sequences are available on request. Chromosomal DNA and plasmid DNA were isolated with commercial kits (Achieve pure DNA cell/tissue, 5 Prime; GenElute Plasmid, Sigma). DNA was amplified using a high-fidelity polymerase (Phusion, New England Biolabs) and cloned using yeast in vivo cloning [29]. All vectors used were previously described [29,30]. The *crp* open reading frame (ORF) of *S. marcescens* was cloned into pMQ131 ($_{ori}$pBBR1) using yeast in vivo recombination [29] and placed under transcriptional control of the *E. coli* P_{lac} promoter, generating pRMQS166 or "p*crp*". A mutation was made in the *crp* gene of pRMQS166, by digestion with Sal1, followed by end polishing using the End-IT kit (Epicenter, Madison, WI, USA), and ligation with T4 DNA ligase (NEB, Beverly MA). The resulting construct, pEJK2 (p*crp-Δ4*), had a 4 base pair deletion in the *crp* gene starting at base pair 217 of the 630 base pair open reading frame; this allele is labeled as *crp-Δ4*. The *cyaA* and *cpdA* genes were cloned into the pMQ131 vector [30] and expressed from the *E. coli* P_{lac} promoter, yielding p*cyaA* and p*cpdA*.

Mariner transposon mutations were generated as previously described using pBT20 [31]. The *crp-1*, *crr-1* and *fimC-4* were previously generated [18,31] and constructed by introduction of plasmid pMQ118 with an internal fragment of these genes, designed to recombine with the chromosomal respective gene, yielding a disruption of the gene. Mutations were verified using PCR.

***2.2.1. Allelic replacement of* crp—**The *crp-Δ4* allele from pEJK2 was cloned into the allelic replacement vector pMQ236 [30], containing a I-SceI meganuclease site for counterselection. After recombination of the pMQ236 + *crp-Δ4* plasmid into the chromosome, a temperature-sensitive I-SceI meganuclease-expressing plasmid, pMQ240, was introduced into the merodiploid strain [30]. The I-SceI meganuclease introduces a double-stranded break in the pMQ236 backbone integrated into the chromosome and thereby selects for either restoration of the *crp* WT gene or replacement of the WT *crp* gene with the *crp-Δ4* allele. Mutations were verified by diagnostic PCR.

The pStvZ3 plasmid was generated as a chromosomal promoter probe to assess transcription of the gene of interest. The full-length *lacZ* gene from pYC2-lacZ (Invitrogen) was introduced into pMQ118 by in vivo cloning. The pStvZ3 plasmid, which is non-replicative in *S. marcescens*, was introduced into various strains by conjugation creating chromosomal *lacZ* fusions at *pigA*. A 611 bp internal fragment of *pigA* was cloned into pStvZ3. Integration of this plasmid creates a transcriptional *lacZ* fusion with *pigA* and causes disruption of *pigA* at base pair 626.

An N-terminal 8-histidine tag (His_8) was added to the *crp* gene and cloned under control of the P_{BAD} promoter on a high-copy ColE1 shuttle vector, pMQ124 using yeast in vivo recombination [30].

2.3. Prodigiosin production assays

Single colonies were inoculated in 5 ml of LB medium ± antibiotics ± cAMP and incubated 16-18 h on a rotary shaker (TC-7, New Brunswick). Prodigiosin was extracted from centrifuged cell pellets with acidified ethanol and levels were determined by measuring absorbance at 534

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

nm, based upon the method of Slater et. al. [32]. Absorbance was read (Beckman DU-70 spectrophotometer) at 534 nm to measure extracted prodigiosin and 600 nm to measure culture turbidity using a 1 cm^2 cuvette, and the ratio was determined. Extracted red pigment had a maximum absorbance at ~535 nm in acidic conditions as expected for prodigiosin (data not shown, [16]). A similar approach was used with M63 agar except that 4 independent cultures of WT and *crp-Δ4* were grown up in LB to saturation, then subcultured and grown to logarithmic phase. Ten μl of each were spotted onto M63 agar plates with different concentrations of glucose (0.2-4.0%). These were incubated at 30°C for 16-19 h, cells were scraped from the plate and cell concentration was measured at A600 nm. Cells were harvested by centrifugation; prodigiosin was extracted and measured at A534 nm. The experiment was repeated three times on different days with similar results.

2.4. Phosphodiesterase and ß-galactosidase (ß-gal) assays

For phosphodiesterase (PDE) activity assays, JM3000 *cpdA::kan* with the appropriate plasmid was grown overnight in LB medium with kanamycin, normalized to A_{600} nm = 1.0; 1 ml of culture medium was pelleted by centrifugation and washed with Tris-Cl reaction buffer (50 mM Tris-Cl, 1 mM $MnCl_2$, pH 8.5). The cells were resuspended in 0.75 ml Tris-Cl reaction buffer and sonicated on ice until clear. The lysate was centrifuged at 16100 × g for 15 min at 4°C and the protein concentration of supernatants was determined by the Bradford assay. PDE activity from 100 μg of protein was measured using the chromogenic phosphodiesterase substrate bis(p-nitrophenyl) phosphate (bis-*p*NPP, Sigma, product number N3002) at a final concentration of 5 mM in Tris-Cl reaction buffer. Reactions were incubated at 37°C for 15 min and yellow color resulting from bis-*p*NPP hydrolysis was measured at A=410 nm with a plate reader (Biotek Synergy 2, Winooski VT).

For ß-gal assays, cultures were grown overnight in LB medium with kanamycin at 30°C, then subcultured (1:100) two times and grown to an A_{600} of 0.1 in order to synchronize cultures in early exponential growth phase. After growth to a desired optical density, culture aliquots were pelleted, washed with Z-buffer, and ß-galactosidase (ß-gal) activity was determined [22]. Lysates were prepared by sonication in Z-buffer and were clarified by centrifugation at 16,100 g for 5 min. Protein concentration from supernatants was determined by Bradford analysis, and the same amount of protein from each sample in a given experiment was added to microtiter plate wells and the volume adjusted to 100 μl with Z-buffer. ONPG (25μl at 4mg/ml) was added as a substrate and allowed to incubate until a yellow color developed. A_{410} readings were taken with a plate reader.

2.5. Protein purification and EMSA analysis

The His_8-CRP protein was generated using P_{BAD}-expressed His_8-CRP in *E. coli*. An overnight culture of *E. coli* harboring the P_{BAD}-His_8-*crp* vector, pRMQS242, was subcultured 1:100 in 250 ml of fresh LB with gentamicin and grown to an A_{600} of 0.4 at 30°C. L-arabinose was added to a final concentration of 0.2%, and incubation was continued for 4 h to induce expression of His_8-CRP. Bacteria were then harvested by centrifugation (4,000 × g for 15 min, 4°C), resuspended in B-Per lysis solution (Pierce) with 1X Halt protease Inhibitor Cocktail (Pierce), and lysed on ice by sonication (ten bursts of 10s at ~20W). The lysate was then centrifuged (4,000 × g for 15 min, 4°C) and His $_8$-CRP purified with IMAC (Pierce HisPur Cobalt Spin Column, Rockford, IL, USA). The purified protein was verified as CRP by immunoblotting with a monoclonal anti-CRP antibody (NeoClone, Madison, WI, USA). The purity of CRP was determined to be >90% as visualized by a Coomassie-stained, SDS-12% polyacrylamide gel electrophoresis (PAGE) and analyzed with Image J software (NIH).

Crude lysates were prepared by growing bacteria overnight in LB broth. Cells (4 ml) were harvested and washed twice with Tris-buffered saline, pH 8 (TBS), and resuspended in TBS

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(0.75 ml) with HALT protease inhibitor cocktail (Pierce). Cell suspensions were sonicated until cleared and centrifuged for 10 min at 16,100 × g at 4°C. Protein concentration was determined from supernatants and used directly in some EMSA reactions.

Labeled DNA amplicons were made with a 5′-biotinylated oligonucleotide primer (Integrated DNA Technologies, Skokie, IL, USA), gel-purified and verified by sequencing. A commercial EMSA kit was employed as specified by the manufacturer (Lightshift Chemiluminescent EMSA kit, Pierce, Rockford IL, USA), using biotinylated target DNA (1-3 ng), purified 8-histidine-tagged CRP (≥50 ng) and poly-dIdC (500 ng), cAMP (500 μM) and non-labeled competitor DNA (0-600 ng) as specified, in a 20 μl reaction. A 10 μl aliquot of the reaction was separated on 5% PAGE, TBE gel (Bio-Rad) with TBE running buffer containing 500 μM cAMP.

2.6. Statistical analysis

GraphPad Prism and Microsoft Excel software was used to determine statistical significance using one-way ANOVA with Tukey's post-test and Student's T-tests as noted.

3. Results

3.1. Glucose inhibits prodigiosin production by S. marcescens

A previous report showed that exogenous glucose inhibited pigment production by *S. marcescens*, leading to the hypothesis that cAMP is a positive regulator of pigment production, as glucose inhibits cAMP production [4]. Inconsistently, we noted that a *cyaA* mutant strain exhibited a strikingly hyperpigmented phenotype, suggesting a negative role for cAMP in regulation of pigment production, as this strain was previously shown to be deficient in intracellular cAMP levels (Table 2). We tested whether glucose also inhibited pigment production in *S. marcescens* strain PIC3611 and found that glucose conferred nearly complete inhibition of wild-type pigment production in LB liquid (Table 2 and Fig. 1).

If glucose inhibits prodigiosin production through reduction of bacterial cAMP levels, then glucose should have no effect upon *crp* mutants, because the CRP protein is required to respond to cAMP. Mutation of *crp* in our strain background eliminated the effect of exogenous cAMP on fimbriae production and expression of *flhD* [18] [33], supporting the notion that this strain is unresponsive to cAMP as a second messenger. We observed that *crp* mutants had a hyperpigment phenotype that was highly sensitive to glucose in LB liquid (Table 2, Fig. 1). This suggests that glucose inhibits prodigiosin production in a cAMP-independent manner.

The glucose effect was also tested in defined minimal medium to ensure it was not an artifact of working with LB medium. There was undetectable prodigiosin production in liquid minimal medium (data not shown); however, pigment production was observed on M63 agar plates. A statistically significant decrease in pigment production was observed for both WT and *crp-Δ4* mutants grown on M63 agar when glucose levels were increased (Table 2). These results support the model showing that glucose inhibits pigment production in a cAMP-independent manner, and suggest that previous studies misinterpreted the glucose effect on prodigiosin as being due to altered cAMP levels.

3.2. Prodigiosin production is dependent upon cAMP-related genes: cyaA, crr *and* crp

Mutation of genes involved in production of cAMP (*crr*, *cyaA*) or cAMP-dependent transcriptional signaling (*crp*) conferred a dramatic increase in red pigment production compared to the wild type (Table 2). We performed experiments to quantify the role of these catabolite repression system genes in pigment production. As noted above for CyaA, the Crr protein of *S. marcescens* is also required for cAMP production in our strain background, as

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

mutants have a severe reduction in intracellular cAMP levels [18]. Prodigiosin levels from stationary phase cultures grown in LB medium were found to be elevated >400% in the *cyaA* strain over wild-type levels (Table 2). This hyperpigment phenotype was complemented by the wild-type *cyaA* gene on a multicopy plasmid (*cyaA-2* + p*cyaA*) (Table 2). A similar trend was observed with different *crp* mutant alleles (*crp-Δ4* and *crp-23*) (Table 2). We were able to complement the hyperpigment phenotype of *crp* mutants with the wild-type *crp* gene in trans (*crp-23* + p*crp*), but not by the same plasmid with a 4 bp deletion in the *crp* gene (*crp-23*+ p*crp-Δ4*) (Table 2). Furthermore, multicopy expression of the wild-type *crp* gene in the *crp-23* mutant (*crp-23* + p*crp*), but not multicopy expression of a mutant *crp* allele (*crp-23* + p*crp-Δ4*) confers pigment levels lower than the wild-type, suggesting that multicopy expression of the functional *crp* gene inhibits prodigiosin production (Table 2).

Further supporting the idea that inactivation of the cAMP-dependent catabolite repression system activates prodigiosin production, we were able to efficiently screen for random transposon-induced mutations in *crp*, *crr*, and *cyaA* genes based upon their hyper-red colony phenotype, e.g. *crp-23* (Table 2, data not shown). Random transposon-induced suppressor mutations of the *crp* hyperpigment phenotype mapped to prodigiosin biosynthetic genes *pigB*, *pigC*, *pigD* and *pigM*, confirming our spectrophotometric analysis (Materials and methods section) indicating that the hyperpigmented phenotype of *crp* mutants was due to elevated levels of prodigiosin rather than some other red pigment (Table 2 and data not shown). The use of multiple mutant alleles and complementation analysis provides strong genetic evidence in support of the hypothesis that prodigiosin is inhibited by cAMP in our strain background.

Insertion mutagenesis of *crp* in the *S. marcescens* strain Nima (ATCC#29632), which has been extensively used to study prodigiosin production [14,38], led to a similar increase in red pigment production, indicating that CRP's impact is not limited to our laboratory strain background (Table 2).

3.3. Prodigiosin production is inhibited by exogenous cAMP

If cAMP negatively regulates prodigiosin production, then growth of bacteria in medium supplemented with cAMP should yield reduced pigment production. A cAMP dose-dependent decrease in pigment production by the WT strain was measured (Fig. 2A). Exogenous cAMP reversed the hyperpigment phenotype of *cyaA* mutants (Fig. 2B) and inhibited prodigiosin production in pigmented *S. marcescens* strains Nima, K904 and CHASM (Table 2), suggesting a conserved regulatory mechanism among diverse strains.

Mutation of *crp* was predicted to render pigment production unresponsive to exogenous cAMP, since CRP is required for cAMP-induced signal transduction. Consistently, we observed no significant change (p=0.46) in prodigiosin levels from the *crp* mutant grown with cAMP at 10 mM (Fig. 2B). Because the *crp* mutant was unaffected, we conclude that the effect of cAMP on pigment production was through the cAMP-CRP signal transduction pathway rather than an unknown physiological effect from the exogenous cAMP. Growth of WT, *cyaA* and *crp* mutants is not decreased by exogenous cAMP [18] (data not shown).

3.4. Multicopy expression of a bona fide cAMP phosphodiesterase gene increases prodigiosin production

The *E. coli* cAMP phosphodiesterase gene, *cpdA*, codes for a protein that hydrolyzes cAMP into 5′-AMP [17]. We predicted that multicopy expression of a known cAMP phosphodiesterase gene would increase pigment production if cAMP negatively regulates prodigiosin production. To do this, we cloned the *cpdA* gene from *E. coli* and placed it under the control of the *E. coli Plac* promoter on a medium-copy pBBR1-based plasmid (p*cpdA*). To

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

ensure that multicopy expression of this gene conferred phosphodiesterase (PDE) activity, an *E. coli cpdA::kan* mutant was transformed using p*cpdA*, and phosphodiesterase activity was measured from the resulting crude lysates. A significant increase in PDE activity was observed in cells bearing multiple copies of *cpdA* compared to the same strain with the empty vector (pMQ131) (Fig. 2C). We found that multicopy expression of *cpdA* in WT *S. marcescens* conferred a significant increase in pigment production (Fig. 2D), without altering growth (data not shown).

A mutant version of *cpdA* (*cpdA-1*) was fortuitously made through PCR errors, with S70F and G80S changes. Multicopy expression of this mutant version conferred an intermediate amount of phosphodiesterase activity upon *E. coli* (Fig. 2C). Multicopy expression of the mutant *cpdA-1* allele in WT *S. marcescens* conferred a moderate significant increase in pigment production (Fig. 2D), correlating PDE activity with pigment production. Another mutant strain with only an S70F mutation was found and had no difference in PDE activity (data not shown); from this result we infer that a G80S change deleteriously impacts CpdA's PDE activity.

3.5. Control of pigA *transcription by cAMP-CRP*

If cAMP inhibits pigment production at the transcriptional level, then exogenous cAMP would be expected to produce a decrease in expression of the prodigiosin biosynthetic operon. To test this prediction, we generated a chromosomal *pigA-lacZ* transcriptional reporter (*pigA* is the first gene in the prodigiosin biosynthetic operon), grew bacteria to early log phase, split the cultures and added cAMP to various concentrations. Bacteria were harvested at A_{600} nm = 5.5 (~12 h) in early stationary phase when *pigA* transcript levels are predicted to be highest, and β-gal levels were determined. Similar to its effect on pigment production, exogenous cAMP reduced wild-type *pigA-lacZ* expression in a significant dose-dependent manner (Fig. 3A).

In the absence of exogenous cAMP, the wild-type strain with the *pigA-lacZ* construct exhibited a culture density-dependent increase in ß-galactosidase activity, in agreement with previous reports (Fig. 3B) [8,10]. In *crp* mutant strains, the amount of *pigA-lacZ*-associated ß-galactosidase activity was ~5-fold higher (A_{600}=4) than WT levels, (Fig. 3B). Two different *crp* mutant alleles were used to support the notion that the phenotype was a result of *crp* mutation.

A previous report speculated that CRP may regulate pigment production, as a predicted CRP-binding site was detected upstream of the pigment biosynthetic operon in *Serratia* sp. *ATCC 39006* [8]. The ATCC 39006 species has the pigment biosynthetic operon located in a different genomic context compared to several analyzed strains of *S. marcescens* [15]. We found that the sequence of the *pigA* promoter from our strain is highly similar to the reported pigment operon promoter from *S. marcescens* strain 274 [15], and neither have a predicted CRP binding site within 400 base pairs of the start codon. Although there was no consensus CRP binding site in the *pigA* promoter region, we performed EMSA assays with purified CRP to determine whether CRP directly binds the *pigA* promoter. We used the *S. marcescens flhD* promoter region as a putative positive control, as we had previously shown that *flhD* expression is positively regulated by cAMP and CRP [33], and the *flhD* promoter region has a predicted CRP binding site [13]. CRP bound DNA upstream of the *flhD* open in vitro (lane 2, Fig. 3C), whereas DNA upstream of *pigA* was not bound (data not shown). As an alternative method to test for CRP binding to *pigA*, we attempted to titrate out CRP-*flhD* promoter binding with excess unlabeled *pigA* promoter DNA. CRP-*flhD* promoter interactions could be inhibited by an excess of unlabelled *flhD* DNA (lane 3-4, Fig. 3C); however, an excess of unlabelled *pigA* promoter DNA was unable to compete for CRP-*flhD* promoter interactions (lane 5-7, Fig. 3C). Lastly, EMSA reactions performed with crude lysates from both WT and a *crp-23* mutant strain both conferred gel shifts of the *pigA* promoter, supporting the idea that the *pigA* promoter can be bound under our experimental conditions and that the gel shift occurs in the absence of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

CRP (lane 2,5, Fig. 3D). Together these experiments support the model indicating that cAMP-CRP negatively regulates the prodigiosin biosynthetic operon in an indirect manner, rather than by directly binding to the *pigA* promoter.

4. Discussion

Here we present a model in which cAMP-CRP negatively regulates prodigiosin production through transcriptional control of the pigment biosynthetic operon. In support of this model, (1) mutation in genes known to be required for full levels of intracellular cAMP in our strain background conferred a large increase in pigment production, and this hyperpigment phenotype could be suppressed by exogenous cAMP; (2) null mutation of the *crp* gene, which is necessary for the cell to respond to cAMP, conferred a significant increase in prodigiosin, and this hyperpigment phenotype could not be reversed by exogenous cAMP; (3) multicopy expression of a functional but not a null mutant version of the *crp* gene reduced pigment production; (4) multicopy expression of a functional bona fide cAMP-PDE gene conferred a significant increase in pigment production; (5) expression of the first gene of the pigment biosynthetic operon (*pigA*) is inhibited by exogenous cAMP, and increased in *crp* mutants. The absence of a CRP binding site in the *S. marcescens pigA* promoter and the negative EMSA studies suggest that CRP indirectly regulates pigment biosynthetic operon expression. This may not be surprising, given the large number of pigment regulators described in *Serratia* sp. ATCC 39006 and *S. marcescens* that likely serve as intermediate regulators, reviewed by Williamson, et al. [39].

Previous reports have suggested a positive regulatory role for cAMP in *Serratia* prodigiosin production. One report showed that glucose severely repressed prodigiosin production in *S. marcescens* and that this inhibition could be reduced by theophylline [4], which is a potent inhibitor of purified cAMP phosphodiesterase activity in vitro [26]. While these are interesting observations, it was not clear whether the exogenous addition of glucose or theophylline had effects on multiple regulatory systems that could lead to altered pigment production. Data presented here also support the hypothesis that glucose inhibits prodigiosin production, but suggest that this is a cAMP-independent phenomenon, as glucose also inhibits pigment production in a *crp* mutant. We are in the process of finding and characterizing genes in this pathway using random mutagenesis.

Another report more strongly suggests that cAMP is a positive regulator of prodigiosin production [40]. In that report, a *S. marcescens* strain lacking adenylate cyclase (CyaA) and cAMP phosphodiesterase activity exhibited reduced pigment production and this deficiency could be rescued by exogenous cAMP, though these findings were not quantified [40]. The differences between the previous study and the current one could be due to strain differences, which are common amongst *S. marcescens* strains [20,31,33]. Phenotypes from the strain used in the former study [40] were not complemented nor were the mutations mapped, so these effects could result from unknown mutations elsewhere in the chromosome. The lack of adenylate cyclase activity in the strain used in the former study [40] could stem from mutation of the *cyaA* gene, the *crr* gene or other possible regulators of cAMP production and pigment. The data presented here show a negative role for cAMP in pigment production by four different strains (Table 2) using defined isogenic strains with mutations in catabolite repression genes to establish this role.

A recent report by Haddix and coworkers provides evidence that prodigiosin has a role in energy spilling [14]. Given the known role of cAMP in the starvation response of *E. coli*, it follows that cAMP production and prodigiosin production would be inversely regulated, as it would be counterproductive to activate an energy spilling mechanism (prodigiosin production) under starvation conditions (elevated cAMP levels). Together, these data support a teleological

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

argument for why cAMP would be directly tied to prodigiosin production under both favorable environmental conditions and starvation conditions. The role of cAMP in inhibition of prodigiosin has been observed in another common soil microorganism, *Streptomyces coelicolor*, suggesting a conserved role [34].

Prodigiosin has potential as a therapeutic anti-cancer agent [27]. Several studies have reported methods to increase prodigiosin production by alteration of media constituents or by cloning of the prodigiosin biosynthetic operon [7,12] [15,35]. Here we have characterized a significant increase in pigment production by mutation of catabolite repression system components, suggesting that this pathway could be exploited in large-scale production of prodigiosin.

Acknowledgments

We acknowledge Pryce Haddix for helpful discussion and the kind gift of strain Nima. This work was supported by the Campbell Laboratory of Ophthalmic Microbiology, the Eye and Ear Foundation of Pittsburgh, a career development award from Research to Prevent Blindness (R.S.), and an NEI Core Grant for Vision Research, EY08098.

References

1. Blizzard JL, Peterson GE. Selective inhibition of proline-induced pigmentation in washed cells of *Serratia marcescens*. J. Bacteriol 1963;85:1136–1140. [PubMed: 14044006]
2. Botsford JL, Harman JG. Cyclic AMP in prokaryotes. Microbiol. Rev 1992;56:100–122. [PubMed: 1315922]
3. Burger SR, Bennett JW. Droplet enrichment factors of pigmented and nonpigmented *Serratia marcescens*: possible selective function for prodigiosin. Appl. Environ. Microbiol 1985;50:487–490. [PubMed: 3901922]
4. Clements-Jewery S. The reversal of glucose repressed prodigiosin production in *Serratia marcescens* by the cyclic 3′5′-adenosine monophosphate inhibitor theophylline. Experientia 1976;32:421–422. [PubMed: 773661]
5. Coulthurst SJ, Kurz CL, Salmond GP. *luxS* mutants of *Serratia* defective in autoinducer-2-dependent ‘quorum sensing’ show strain-dependent impacts on virulence and production of carbapenem and prodigiosin. Microbiology 2004;150:1901–1910. [PubMed: 15184576]
6. Crasnier M. Cyclic AMP and catabolite repression. Res. Microbiol 1996;147:479–482. [PubMed: 9084758]
7. Dauenhauer SA, Hull RA, Williams RP. Cloning and expression in *Escherichia coli* of *Serratia marcescens* genes encoding prodigiosin biosynthesis. J. Bacteriol 1984;158:1128–1132. [PubMed: 6233267]
8. Fineran PC, Everson L, Slater H, Salmond GP. A GntR family transcriptional regulator (PigT) controls gluconate-mediated repression and defines a new, independent pathway for regulation of the tripyrrole antibiotic, prodigiosin, in *Serratia*. Microbiology 2005;151:3833–3845. [PubMed: 16339930]
9. Fineran PC, Slater H, Everson L, Hughes K, Salmond GP. Biosynthesis of tripyrrole and beta-lactam secondary metabolites in *Serratia*: integration of quorum sensing with multiple new regulatory components in the control of prodigiosin and carbapenem antibiotic production. Mol. Microbiol 2005;56:1495–1517. [PubMed: 15916601]
10. Fineran PC, Williamson NR, Lilley KS, Salmond GP. Virulence and prodigiosin antibiotic biosynthesis in *Serratia* are regulated pleiotropically by the GGDEF/EAL domain protein, PigX. J. Bacteriol 2007;189:7653–7662. [PubMed: 17766413]
11. Gerber NN. Prodigiosin-like pigments. CRC Crit. Rev. Microbiol 1975;3:469–485. [PubMed: 1095305]
12. Giri AV, Anandkumar N, Muthukumaran G, Pennathur G. A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. BMC Micro 2004;4

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

13. Givskov M, Eberl L, Christiansen G, Benedik MJ, Molin S. Induction of phospholipase- and flagellar synthesis in *Serratia liquefaciens* is controlled by expression of the flagellar master operon *flhD*. Mol. Microbiol 1995;15:445–454. [PubMed: 7783615]
14. Haddix PL, Jones S, Patel P, Burnham S, Knights K, Powell JN, LaForm A. Kinetic analysis of growth rate, ATP, and pigmentation suggests an energy-spilling function for the pigment prodigiosin of *Serratia marcescens*. J. Bacteriol 2008;190:7453–7463. [PubMed: 18805986]
15. Harris AK, Williamson NR, Slater H, Cox A, Abbasi S, Foulds I, Simonsen HT, Leeper FJ, Salmond GP. The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation. Microbiology 2004;150:3547–3560. [PubMed: 15528645]
16. Hubbard R, Rimington C. The biosynthesis of prodigiosin, the tripyrrylmethene pigment from *Bacillus prodigiosus* (*Serratia marcescens*). Biochem. J 1950;46:220–225. [PubMed: 16748664]
17. Imamura R, Yamanaka K, Ogura T, Hiraga S, Fujita N, Ishihama A, Niki H. Identification of the *cpdA* gene encoding cyclic 3′,5′-adenosine monophosphate phosphodiesterase in *Escherichia coli*. J. Biol. Chem 1996;271:25423–25429. [PubMed: 8810311]
18. Kalivoda EJ, Stella NA, O'Dee DM, Nau GJ, Shanks RM. The cAMP-dependent catabolite repression system of *Serratia marcescens* mediates biofilm formation through regulation of type 1 fimbriae. Appl. Environ. Microbiol 2008;74:3461–3470. [PubMed: 18424546]
19. Kolb A, Busby S, Buc H, Garges S, Adhya S. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem 1993;62:749–795. [PubMed: 8394684]
20. Labbate M, Zhu H, Thung L, Bandara R, Larsen MR, Willcox MDP, Givskov M, Rice SA, Kjelleberg S. Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. J. Bacteriol 2007;189:2702–2711. [PubMed: 17237163]
21. Levy S, Zeng GQ, Danchin A. Cyclic AMP synthesis in *Escherichia coli* strains bearing known deletions in the pts phosphotransferase operon. Gene 1990;86:27–33. [PubMed: 2155859]
22. Miller, JH. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1972.
23. Miller VL, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J. Bacteriol 1988;170:2575. [PubMed: 2836362]
24. Montaner B, Navarro S, Pique M, Vilaseca M, Martinell M, Giralt E, Gil J, Perez-Tomas R. Prodigiosin from supernatent of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. Br. J. Pharmacol 2000;131:585–593. [PubMed: 11015311]
25. Morohoshi T, Shiono T, Takidouchi K, Kato M, Kato N, Kato J, Ikeda T. Inhibition of Quorum Sensing in *Serratia marcescens* AS-1 by the Synthetic Analogs of N-Acylhomoserine Lactone. Appl. Environ. Microbiol. 2007
26. Okabayashi T, Ide M. Cyclic 3′,5′-nucleotide phosphodiesterase of *Serratia marcescens*. Biochim. Biophys. Acta 1970;220:116–123. [PubMed: 4319606]
27. Perez-Tomas R, Montaner B, Llagostera E, Soto-Cerrato V. The prodigiosins, proapoptotic drugs with anticancer properties. Biochem. Pharmacol 2003;66:1447–1452. [PubMed: 14555220]
28. Rosenberg M, Blumberger Y, Judes H, Bar-Ness R, Rubinstein E, Mazor Y. Cell surface hydrophobicity of pigmented and nonpigmented clinical *Serratia marcescens* strains. Infect. Immun 1986;51:932–935. [PubMed: 3512440]
29. Shanks RM, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. Appl. Environ. Microbiol 2006;72:5027–5036. [PubMed: 16820502]
30. Shanks RM, Kadouri DE, MacEachran DP, O'Toole GA. New yeast recombineering tools for bacteria. Plasmid 2009;62:88–97. [PubMed: 19477196]
31. Shanks RM, Stella NA, Kalivoda EJ, Doe MR, M. ODD, Lathrop KL, Guo FL, Nau GJ. A *Serratia marcescens* OxyR homolog mediates surface attachment and biofilm formation. J. Bacteriol 2007;189:7262–7272. [PubMed: 17675374]
32. Slater H, Crow M, Everson L, Salmond GP. Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol. Microbiol 2003;47:303–320. [PubMed: 12519208]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

33. Stella NA, Kalivoda EJ, O'Dee DM, Nau GJ, Shanks RM. Catabolite repression control of flagellum production by *Serratia marcescens*. Res. Microbiol 2008;159:562–568. [PubMed: 18718529]
34. Süsstrunk U, Pidoux J, Taubert S, Ullmann A, Thompson CJ. Pleiotropic effects of cAMP on germination, antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. Mol. Microbiol 1998;30:33–46. [PubMed: 9786183]
35. Thomson NR, Crow MA, McGowan SJ, Cox A, Salmond GP. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. Mol. Microbiol 2000;36:539–556. [PubMed: 10844645]
36. Vining LC. Functions of secondary metabolites. Annu Rev Microbiol 1990;44
37. Williams RP, Gott CL, Qadri SM, Scott RH. Influence of temperature of incubation and type of growth medium on pigmentation in *Serratia marcescens*. J. Bacteriol 1971;106:428–443.
38. Williams RP, Green JA, Rappo-Port DA. Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of prodigiosin. J. Bacteriol 1956;71:115–120. [PubMed: 13286239]
39. Williamson NR, Fineran PC, Leeper FJ, Salmond GP. The biosynthesis and regulation of bacterial prodiginines. Nat. Rev. Microbiol 2006;4:887–899. [PubMed: 17109029]
40. Winkler U, Scholle H, Bohne L. Mutants of *Serratia marcescens* lacking cyclic nucleotide phosphodiesterase activity and requiring cyclic 3′,5′-AMP for the utilization of various carbohydrates. Arch. Microbiol 1975;104:189–196. [PubMed: 168832]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

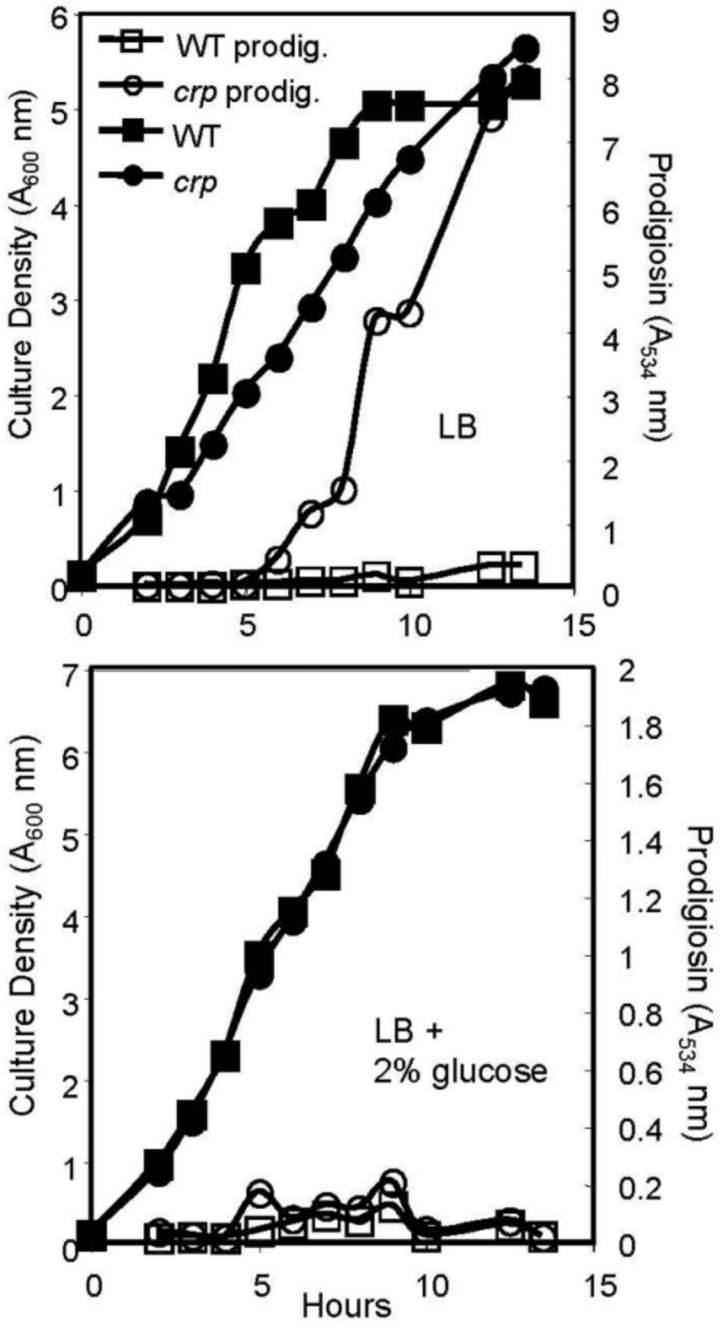

Fig. 1.

Mutation of *crp* confers a hyperpigment phenotype and prodigiosin production is inhibited by glucose. (Top) Growth and prodigiosin production by WT (*fimC-4*) and an isogenic *crp* (*crp-1 fimC-2*) mutant strain in LB medium. The *fimC* mutation is used to maintain a homogenous culture, as *crp* mutants aggregate and form robust biofilms on test tubes that could complicate growth analysis. The average of 4 independent replicates per strain are shown. (Bottom) Growth and prodigiosin production by WT (*fimC-4*) and an isogenic *crp* (*crp-1 fimC-2*) mutant strain in LB medium supplemented with 2% glucose. The averages of 4 independent replicates per strain are shown. These experiments were performed on different days with the same trend.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

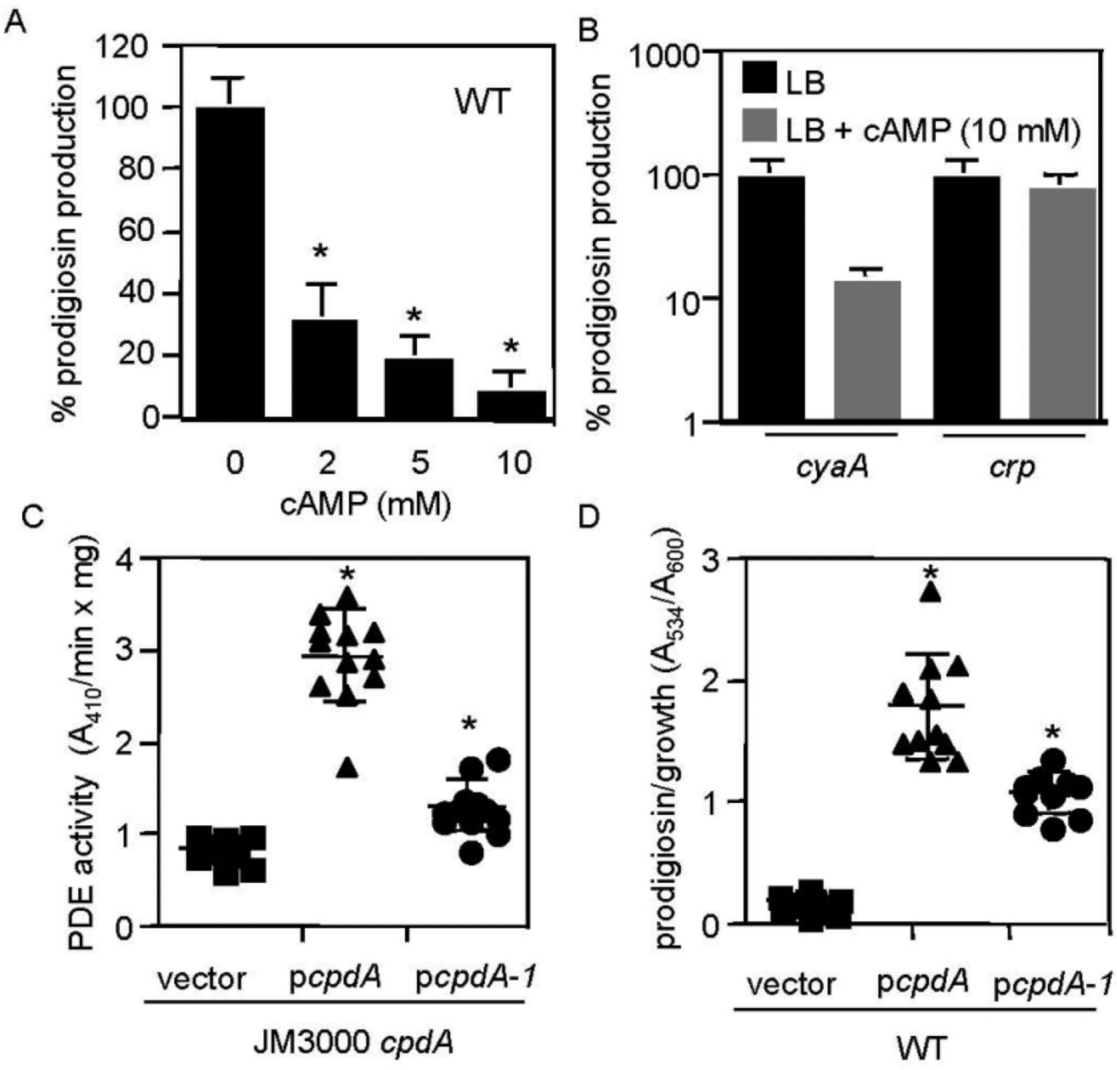

Fig. 2.

Exogenous cAMP inhibits prodigiosin production, whereas multicopy expression of a bona fide cAMP-PDE gene confers a hyperpigment phenotype. (A) Exogenous cAMP in LB medium elicits a dose-dependent reduction in prodigiosin production from the WT strain. Prodigiosin levels normalized by culture density are shown as a percentage of WT without exogenous cAMP. This experiment shows the average of 11-12 independent replicates per cAMP concentration from three experiments performed on different days. (B) Prodigiosin levels with respect to culture density in the presence or absence of cAMP (10 mM) in LB medium. Prodigiosin production by the *cyaA*, but not *crp* mutant is sensitive to exogenous cAMP. This experiment shows the average of 6 independent replicates per genotype; the experiment was performed two times on different days. (C) Phosphodiesterase (PDE) activity from crude lysates of a *cpdA* mutant of *E. coli* with an empty pBBR1-based vector, the vector expressing WT *cpdA* from *E. coli* (p*cpdA*), or a mutant version (p*cpdA-1*) using bis-*p*NPP as a substrate. The WT *cpdA* gene confers PDE activity, whereas the p*cpdA-1* mutant confers an intermediate phenotype, indicating partial function. The data are from 10-12 independent replicates from two separate experiments performed on different days. Experiments were performed in LB medium. (D) Prodigiosin production by WT *S. marcescens* bearing an empty pBBR1-based vector, the vector expressing WT *cpdA* from the *E. coli* P_{lac} promoter (p*cpdA*),

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

or a partial-function mutant version (p*cpdA-1*). The *cpdA* gene confers a hyperpigment phenotype to the WT, and the p*cpdA-1* mutant confers an intermediate phenotype. The data are from n≥10 independent replicates from two separate experiments performed on different days. Experiments were performed in LB medium. Asterisk = statistically significant difference from prodigiosin levels achieved without cAMP ($p<0.05$) by one-way ANOVA with the Tukey post-test. Error bars = one standard deviation.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

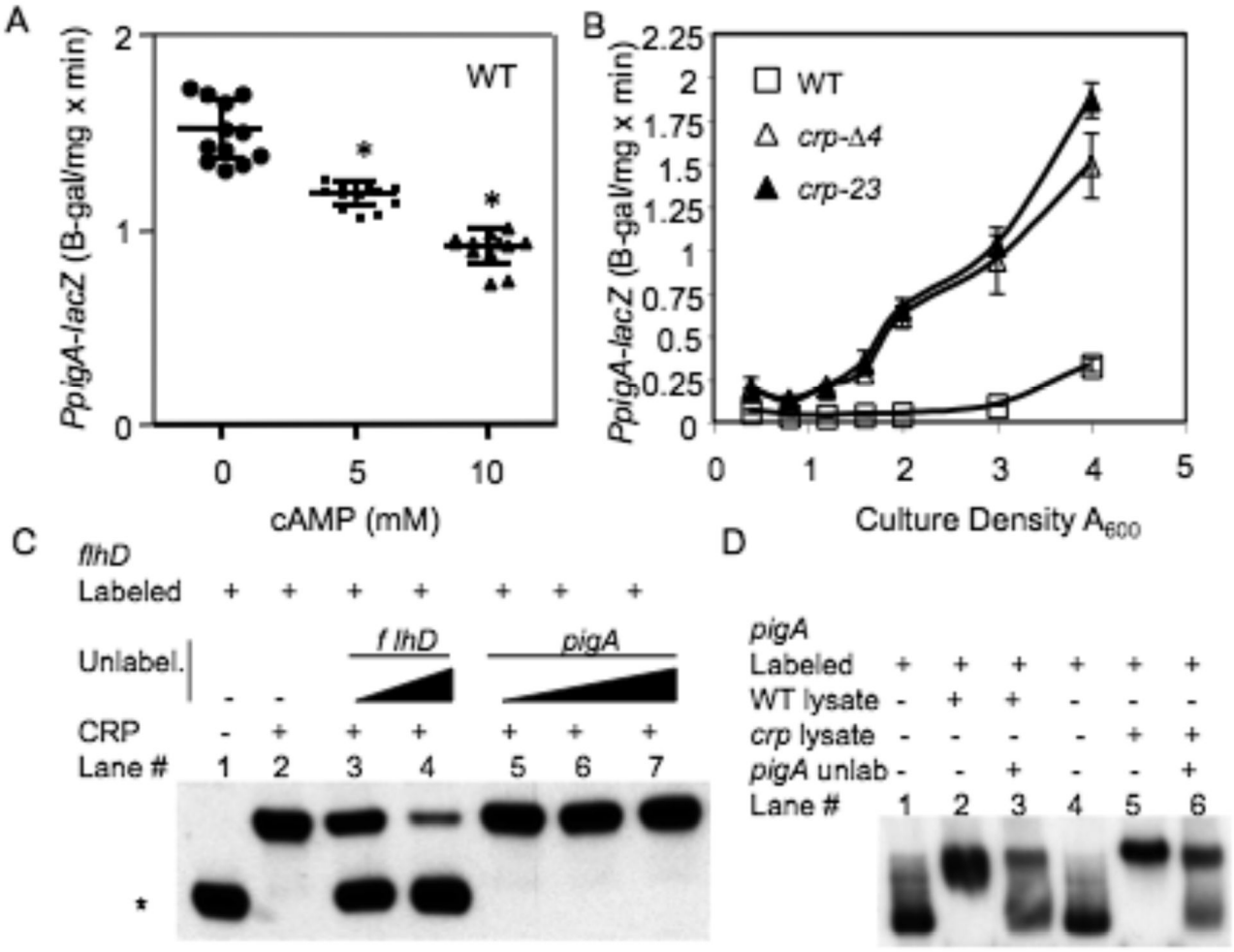

Fig. 3.

A chromosomal *pigA-lacZ* fusion is regulated by CRP and exogenous cAMP. (A) Exogenous cAMP in LB medium elicits a dose-dependent reduction in chromosomal *pigA-lacZ* ß-galactosidase activity at A_{600}=5.5 in a WT background. This experiment shows the average of 12 independent replicates per cAMP concentration, performed on two different days. (B) ß-galactosidase activity as expressed from the chromosomal *pigA* promoter with respect to culture density in LB medium. This experiment shows the average of 3 independent replicates per genotype, the experiment was performed two times on different days with similar results. Experiments were performed in LB medium. (C) Competitive EMSA to determine the ability of unlabeled DNA to compete with His_8-CRP-*flhD* interactions. Biotin-labeled *flhD* promoter DNA (1 ng) was incubated with His_8-CRP at 0 ng (lane 1) or 50 ng (lanes 2-7) per reaction. Unlabeled promoter fragments of *flhD* (lanes 3-4) and *pigA* (lanes 5-7) were incubated in the specified binding reactions at 0 ng (lanes 1-2), 50 ng (lane 3), 200 ng (lanes 4 - 5), 400 ng (lane 6) or 600 ng (lane 7). The asterisk indicates the migration of unbound labeled-*flhD* promoter DNA, signifying successful competitive inhibition of His_8-CRP-*flhD* promoter interactions (lanes 3-4). (D) EMSA analysis with crude lysates. Biotin-labeled *pigA* promoter DNA (2 ng, lanes 1-6) was incubated with crude lysates from WT (lanes 2-3, 10 μg of protein) or *crp-23* cultures (lanes 5-6, 8 μg of protein). Lanes 1 and 4 have no protein added. Unlabeled *pigA* promoter DNA (1100 ng) was added (lanes 3 and 6) to compete for binding. Asterisk =

statistically significant difference from prodigiosin levels achieved without cAMP ($p<0.05$) by one-way ANOVA with the Tukey post-test. Error bars = one standard deviation.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table 1

Strains and plasmids

Strain or plasmid	Description	Reference or source
S. cerevisiae		
InvSc1	*MAT**a**/MATa leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 his3-Δ1/his3-Δ1*	Invitrogen
E. coli		
SM10 λpir	*thi thr leu tonA lacY supE recA::RP4-2Tc::Mu pir*	[23]
S17-1 λpir	*thi pro hsdR hsdM*$^{+}$ *ΔrecA* RP4-2::TcMu-Km::*Tn7 pir*	[23]
JM3000 *cpdA::kan*	*cpdA* null mutation	*E. coli* Genetic Stock Center
S. marcescens		
CMS376 WT	PIC strain number 3611	Presque Isle Cultures
CMS737	*fimC-4*	[18]
CMS641	*crp-1 fimC-2*	[18]
CMS524	*cyaA-2* (transposon mutation)	[18]
CMS772	*crr-1* (insertion mutation)	[18]
CMS786	*crp-23* (transposon mutation)	This study
CMS592B	*pigB::Tnmariner*	This study
CMS613	*crp-1* (insertion mutation)	[18]
CMS794	*crp-1 pigB::Tnmariner*	This study
CMS1687	*crp-Δ4* (deletion mutation)	This study
CHASM	pigmented environmental isolate	This study
K904	pigmented ocular clinical isolate	This study
Nima	ATCC#29632	Pryce Haddix
CMS1812	Nima *crp-1*	This study
Plasmids		
pMQ117	$_{ori}$pSC101^{ts}, *aacC1*, oriT, P_{lac}-*lacZa*, URA3, *CEN6/ARSH4*	[30]
pMQ118	*nptII*, *rpsL*, oriT, $_{ori}$R6K, URA3, *CEN6/ARSH4*	[30]
pMQ124	$_{ori}$iColE1, P_{BAD}-*lacZa*, $_{ori}$pRO1600, $_{ori}$p15a, oriT, URA3, *CEN6/ARSH4*	[30]
pMQ131	$_{ori}$pBBR1, *aphA-3*, oriT, URA3, *CEN6/ARSH4*	[30]
pStvZ3	$_{ori}$R6K *lacZ nptII* promoter probe, oriT, URA3, *CEN6/ARSH4*	This study
pMQ236	pMQ118 with I-SceI site	[30]
pMQ240	pMQ117 + I-SceI meganuclease, oriT	[30]
pRMQS157/p*cyaA*	pMQ131 + *cyaA*	[18]
pRMQS166/p*crp*	pMQ131 with *crp* ORF	This study
pEJK2/p*crp-Δ4*	pRMQS166 with *crp-Δ*4	This study
pEJK3/p*cpdA*	pMQ131 + *cpdA*	This study
pEJK4/p*cpdA-1*	pMQ131 + *cpdA*-1 (S70F,G80S)	This study
pRMQS242	pMQ124 + His_8-*crp*	This study
pRMQS260	pMQ236 + *crp-Δ4*	This study
pRMQS268	pStvZ3 + *pigA* internal fragment	This study

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Table 2

Effect of glucose, cAMP and mutations upon prodigiosin production.

Strain[a]	Medium[b]	Prodigiosin[c]
WT	LB	0.14 ± 0.02
WT	LB + glucose (2% w/v)	<0.01
crp-23	LB	0.88 ± 0.18
crp-23	LB + glucose (2% w/v)	<0.01
WT	M63 + glucose (0.4% w/v)	0.47±0.11
WT	M63 + glucose (2% w/v)	0.16±0.03*
WT	M63 + glucose (4% w/v)	0.16±0.02*
crp-Δ4	M63 + glucose (0.4% w/v)	1.20±0.23
crp-Δ4	M63 + glucose (2% w/v)	0.23±0.06*
crp-Δ4	M63 + glucose (4% w/v)	0.17±0.07*
cyaA-2	LB	0.63 ± 0.03
crr-1	LB	0.48 ± 0.01
crp-Δ4	LB	0.92 ± 0.16
WT + pMQ131 (vector)	LB	0.13 ± 0.03
WT + p*cyaA*	LB	0.15 ± 0.02
cyaA-2 + pMQ131 (vector)	LB	0.46 ± 0.04
cyaA-2 + p*cyaA*	LB	0.11 ± 0.02
crp-23 + pMQ131 (vector)	LB	1.19 ± 0.22
crp-23 + p*crp*	LB	<0.01
crp-23 + p*crp-Δ4*	LB	1.03 ± 0.03
pigB::Tnmariner	LB	<0.01
crp-1 pigB::Tnmariner	LB	<0.01
Nima	LB	0.30 ± 0.03
Nima *crp-1*	LB	3.93 ± 0.59
Nima	LB + cAMP (10 mM)	0.05 ± 0.01
K904	LB	0.33 ± 0.06
K904	LB + cAMP (10 mM)	0.13 ± 0.01
CHASM	LB	0.06 ± 0.01
CHASM	LB + cAMP (10 mM)	0.02 ± 0.01

[a] All strains in CMS376 background unless noted. All experiments were performed in LB liquid medium.

[b] Experiments with LB were done in liquid medium, M63 were with solid medium.

[c] A_{534}/A_{600}, measured at 16-18 h; for LB experiments, a representative experiment is shown with the average of 4 independent biological replicates per data point ± one standard deviation. For M63 experiments, data is an average of 8 independent replicates from two separate experiments per strain ± one standard deviation.