Polish Journal of Microbiology
2019, Vol. 68, No 1, 43–50
https://doi.org/10.21307/pjm-2019-005

ORIGINAL PAPER

# Thermoregulation of Prodigiosin Biosynthesis by *Serratia marcescens* is Controlled at the Transcriptional Level and Requires HexS

ERIC G. ROMANOWSKI, KARA M. LEHNER, NATALIE C. MARTIN, KRIYA R. PATEL, JAKE D. CALLAGHAN, NICHOLAS A. STELLA and ROBERT M.Q. SHANKS*

Charles T. Campbell Laboratory of Ophthalmic Microbiology, Department of Ophthalmology, University of Pittsburgh, Pittsburgh PA

Submitted 21 August 2018, revised 31 October 2018, accepted 5 November 2018

Abstract

Several biotypes of the Gram-negative bacterium *Serratia marcescens* produce the tri-pyrole pigment and secondary metabolite prodigiosin. The biological activities of this pigment have therapeutic potential. For over half a century it has been known that biosynthesis of prodigiosin is inhibited when bacteria are grown at elevated temperatures, yet the fundamental mechanism underlying this thermoregulation has not been characterized. In this study, chromosomal and plasmid-borne *luxCDABE* transcriptional reporters revealed reduced transcription of the prodigiosin biosynthetic operon at 37°C compared to 30°C indicating transcriptional control of pigment production. Moreover, induced expression of the prodigiosin biosynthetic operon at 37°C was able to produce pigmented colonies and cultures demonstrating that physiological conditions at 37°C allow prodigiosin production and indicating that post-transcriptional control is not a major contributor to the thermoregulation of prodigiosin pigmentation. Genetic experiments support the model that the HexS transcription factor is a key contributor to thermoregulation of pigmentation, whereas CRP plays a minor role, and a clear role for EepR and PigP was not observed. Together, these data indicate that thermoregulation of prodigiosin production at elevated temperatures is controlled largely, if not exclusively, at the transcriptional level.

Key words: secondary metabolite, regulation, pigment, prodigiosin, bacteria, transcription factor

## Introduction

Microbial secondary metabolites include crucial medicines such as antibiotics and anti-inflammatory compounds (Vining 1990). Secondary metabolites influence microbial physiology and behaviors, for example, through regulation of outer membrane vesicle formation (Hoefler et al. 2017), quorum sensing signaling (Waters and Bassler 2005; Barriuso et al. 2018), cellular motility (Daniels et al. 2004), metal acquisition (Demain and Fang 2000; Khan et al. 2018), and spore formation (Demain and Fang 2000).

Some of the most studied secondary metabolites are pigments such as violacein and prodigiosin (Williamson et al. 2006; Choi et al. 2017). Both of these pigments are antimicrobial and therefore likely contribute to microbe-microbe interactions (Danevcic et al. 2016a; Danevcic et al. 2016b; Im et al. 2017; Hage-Hulsmann et al. 2018). This study focuses on the regulation of prodigioisin, a red tri-pyrole pigment made by *Serratia* species and a handful of other organisms. While the biological role for prodigiosin is not clearly elucidated, the Haddix group has provided evidence suggesting that prodigiosin is used to finely tune internal energy levels within the bacterium (Haddix et al. 2008; Haddix and Shanks 2018). With respect to mammalian cells, prodigiosin has multiple biological activities including anticancer and autophagy modulating properties (Cheng et al. 2017; Klein et al. 2017; Lin et al. 2017; Klein et al. 2018). Therefore, a thorough understanding of how this secondary metabolite is regulated can be useful for industrial prodigiosin production as well as giving insight into how the organism interacts with the environment.

A variety of transcription factors and environmental stimuli have been shown to influence prodigiosin

* Corresponding author: R.M.Q. Shanks, Department of Ophthalmology, University of Pittsburgh, Pittsburgh, PA; **e-mail: shanksrm@upmc.edu**

biosynthesis (Williamson et al. 2006). Among the earliest described is thermoregulation of prodigiosin production by *S. marcescens* (Blizzard and Peterson 1963; Williams et al. 1965; Tanaka et al. 2004). At 37°C pigment biosynthesis is strongly inhibited, whereas it is synthesized at temperatures of ~32°C and below. In this study we used a molecular genetic approach to investigate fundamental questions of whether prodigiosin is thermoregulated at the transcriptional level or at the post-transcriptional level and what transcription factors are involved in this process. We conclude that for *S. marcescens* strain PIC3611, thermoregulation is controlled primarily at the transcriptional level and this requires the HexS transcription factor.

## Experimental

### Materials and Methods

**Bacterial strains and growth.** Bacterial strains are listed in Table I. Bacteria were grown in LB broth (Bertani 1951), supplemented with antibiotics when appropriate: tetracycline (10 μg/ml), kanamycin (100 μg/ml), carbenicillin (50 μg/ml), and gentamicin (10 μg/ml) for *S. marcescens* and ampicillin (100 μg/ml) and kanamycin (50 μg/ml) for *Escherichia coli*. For liquid broth, bacteria were aerated using a TC-7 tissue culture roller (New Brunswick Inc). Conjugation was used to transform bacteria as follows. Aliquots (500 μl) of donor and acceptor overnight cultures were pelleted by centrifugation, combined, spotted on an LB agar plate, incubated for ~18 hours, and streaked for single colonies on selective medium. Macrocolonies were generated by spotting 10 μl of culture, which was grown overnight in LB broth, on LB agar plates and incubating at 30 and 37°C for 24 hours.

**Molecular biology.** Plasmids were made using yeast homologous recombination as previously described (Shanks et al. 2006; Shanks et al. 2009). Primer sequences are available upon request. A marnier transposon delivery plasmid with a promoterless *luxCDABE* (*lux*) reporter was derived from pSC189 (Chiang and Rubin 2002) with yeast replication machinery from pMQ132 (Shanks et al. 2009) and the *lux* operon from pGRL1 (Benedetti et al. 2012). The resulting plasmid, pMQ690, was moved into *S. marcescens* by conjugation. Six pigmentless colonies were chosen from approximately 5000 transposon mutants and these were analyzed for *lux* operon expression using a luminometer (Perkin Elmer Wallac 1450-021). Two of the isolates generated light due to the orientation of the transposon in the genome and the mutations were mapped and the insertion junction was sequenced as previously described (Chiang and Rubin 2002). The two insertions mapped within the *pig* operon which rendered the isolates unable to make pigment, and oriented downstream of the *pig* promoter so that light can be measured as a reporter of transcription.

To make *pig* promoter reporter plasmid, pMQ713, the *pig* promoter from strain PIC3611 was cloned upstream of the *lux* operon on an $ori_{pBBR1}$-based low copy plasmid, pMQ670 (R. Shanks, to be described elsewhere) as noted above. This episomal plasmid was used to transform a variety of strains described herein by conjugation.

To make the *lac* promoter reporter plasmid, pMQ99, the $P_{BAD}$-*gfp* portion of the plasmid pMQ72 was replaced

Table I
Strains and plasmids used in this study.

| Strain | Description | Source |
|---|---|---|
| PIC3611 | Wild-type *Serratia marcescens* | Presque Isle Cultures |
| CMS1687 | PIC3611 *crp-Δ4* mutant | (Kalivoda et al. 2010) |
| CMS2097 | PIC3611 *eepR* mutant | (Stella et al. 2015) |
| CMS2922 | PIC3611 *hexS* mutant | (Shanks et al. 2013) |
| CMS4891 | PIC3611 *pigB::tn-lux* pigment operon reporter | this study |
| CMS4892 | PIC3611 *pigF::tn-lux* pigment operon reporter | this study |
| Plasmid | Relevant information | Source |
| pMQ99 | $P_{lac}$-*hcred* shuttle vector | this study |
| pMQ132 | pBBR1-based shuttle vector | (Shanks et al. 2009) |
| pMQ200 | oriR6K-based suicide plasmid with PBAD | (Shanks et al. 2009) |
| pMQ221 | pMQ132 with *pigP* gene | (Shanks et al. 2013) |
| pMQ262 | pMQ200 with *pigAB'* | (Kadouri and Shanks, 2013) |
| pMQ364 | pMQ132 with *eepR* gene | (Stella et al. 2015) |
| pMQ690 | promoterless-*luxCDABE* transposon delivery plasmid | this study |
| pMQ713 | *pig* promoter-*luxCDABE* transcriptional fusion | this study |

with $P_{lac}$-*hcred* using homologous recombination (Shanks et al. 2006; Shanks et al. 2009).

**Reporter analysis and prodigiosin assay.** Bacteria were grown for 18–20 hours in LB medium with aeration and the culture density was read at 600 nm with a SpectraMax M3 spectrophotometer using a 1 cm cuvette. Luminescence was measured from 150 µl aliquot of culture in a black, opaque, 96-well plate with a luminometer as noted above. The ratio of luminescence arbitrary units to $OD_{600}$ was calculated as relative luminescence units (RLU). For HcRed fluorescence, a BioTek Synergy 2 plate reader was used to read fluorescence and optical density ($OD_{600}$) of 150 µl samples of overnight cultures. The ratio of fluorescence to optical density was reported as relative fluorescence units (RFU).

To measure prodigiosin from macrocolonies, colonies were scraped from agar plates using a toothpick and suspended in 1.2 ml of saline. A 0.2 ml sample was used to measure $OD_{600}$ in a 1 cm cuvette, and the remaining 1 ml was pelleted by centrifugation, prodigiosin was extracted using acidified ethanol as previously described (Slater et al. 2003), and prodigiosin was measured using a spectrophotometer at 534 nm.

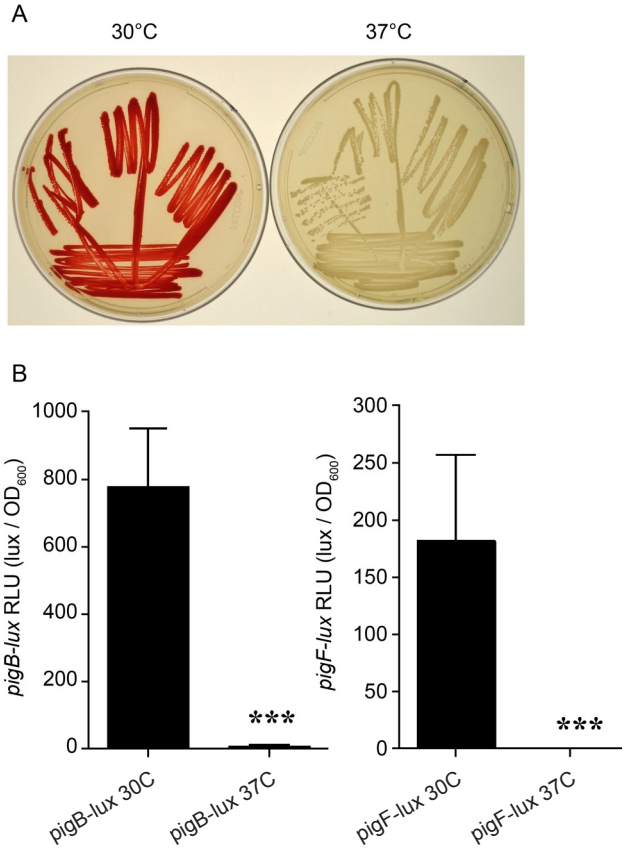


Fig. 1. Thermoregulation of pigmentation by *S. marcescens* is controlled at the transcriptional level.

A. Pigment production of the wild-type strain PIC3611 grown for 18 h at 30°C and 37°C streaked for single colonies on an LB agar plate. B. Thermoregulation of *pig* expression as measured by transposon-borne *luxCDABE* reporters integrated in *pigB* and *pigF* genes. Asterisks indicate a significant difference by Student's T-test ($p < 0.001$, n = 6). Means and standard deviations are shown.

## Results

**Transcriptional control of pigment biogenesis.** Typical of pigmented *S. marcescens* strains, strain PIC3611 does not generate prodigiosin when grown at 37°C but does so at lower temperatures, such as 30°C and below (Fig. 1A). To analyze transcription from the pigment biosynthetic operon (*pig*), we decided to make a *luxCDABE* (*lux*) reporter strain. To achieve this, we generated a promoterless *lux* mariner transposon delivery plasmid and introduced it into the chromosome of *S. marcescens* strain PIC3611. Following transposon mutagenesis, pigmentless colonies were screened with a luminometer for light production after growth at 30°C. Two isolates were found, one mapped to *pigB* (bp 1730) and another to *pigF* (bp 1074) in the *pig* operon. Cultures of these two strains were grown with aeration at 30°C and 37°C, and light production was measured (Fig. 1B). After 18 hours of growth in LB medium, there was a significantly higher level of the signal from both cultures incubated at 30°C, compared to the same strains grown at 37°C. A similar pattern was observed for both *pigB* and *pigF-lux* isolates, although there was a higher level of expression from the *pigB* insertion. This result suggests that temperature regulation of pigment production is, at least in part, controlled at the transcriptional level.

To test whether induced *pig* operon transcription at 37°C could restore prodigiosin biosynthesis, the chromosomal *pigA-N* operon was placed under control of the inducible $P_{BAD}$ operon of wild-type strain PIC3611 by integration of plasmid pMQ262. Prodigiosin biosynthesis of the resulting strain was tightly regulated by addition of L-arabinose to the growth medium. In the absence of L-arabinose, no visible pigmentation was observed, even at 30°C (Fig. 2A). When L-arabinose was added, pigmentation increased in a dose dependent manner at 30°C (Fig. 2A). When tested at 37°C, L-arabinose was able to induce pigmentation in the WT with $P_{BAD}$ promoting *pig* expression (Fig. 2B). Arabinose (2 mM) added to the wild-type strain without pMQ262 did not alter pigmentation (data not shown). This result suggests that transcription of the *pigA-N* operon is sufficient to produce pigmentation even at non-permissive temperatures. This suggests that lack of *pig* operon transcription at 37°C rather than unknown mechanism(s) of post-transcriptional control are responsible for the pigmentation defect at 37°C.

**HexS is necessary to inhibit pigmentation at 37°C.** The CRP and HexS transcription factors are

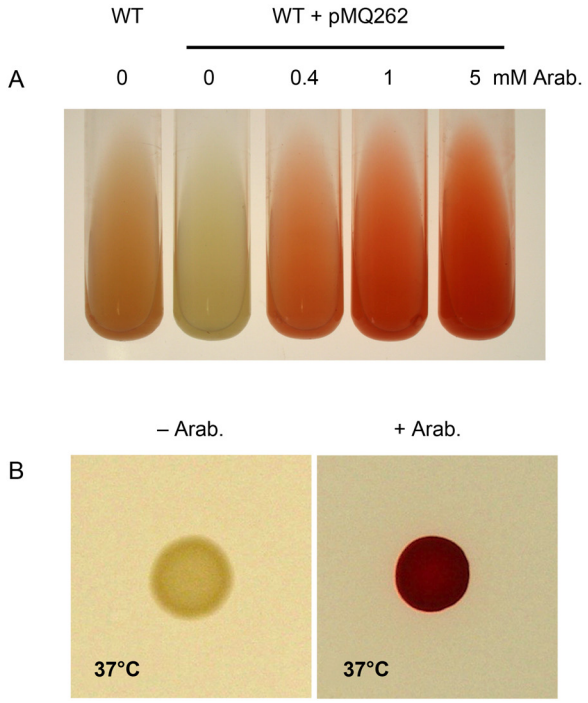


Fig. 2. Induction of *pig* operon at 37°C enables prodigiosin production.

A. Pigment production at 30°C in liquid culture after 18 h of growth at 30°C. The wild-type strain was modified by recombination of pMQ262 through which an L-arabinose-inducible promoter replaces the *pig* promoter. B. Induced expression of *pig* at 37°C enabled expression of prodigiosin demonstrates that prodigiosin can be made at 37°C when the *pig* operon is expressed using pMQ262. Images show representative macrocolonies (see materials and methods).

potent inhibitors of secondary metabolite biosynthesis in strain PIC3611 and other strains, such that *crp* and *hexS* mutants are more highly pigmented than the isogenic wild-type strains (Kalivoda et al. 2010; Stella et al. 2012; Stella and Shanks 2014; Shanks et al. 2017b). It was hypothesized that HexS and CRP were responsible for inhibiting the prodigiosin production at 37°C. To test a role for these transcription factors in thermoregulation, we used well-defined strains with *crp* and *hexS* mutations in the PIC3611 strain background. The prodigiosin phenotypes of these specific strains were previously complemented in trans with the respective genes on plasmids (Kalivoda et al. 2010; Stella and Shanks 2014). The *crp* mutant was only slightly pigmented at 37°C (Fig. 3A), whereas the *hexS* mutant was pigmented at both 30°C and 37°C (Fig. 3B). Pigment from the bacteria on agar plates was measured (Fig. 3C). The *hexS* mutant produced significantly more prodigiosin at 37°C than either wild type or the *crp* mutant. The *crp* mutant did have a ~7.5-fold increase over wild-type prodigiosin levels, but the difference was not significant. Additionally, in liquid cultures grown at 37°C, the *hexS* but not the *crp* mutant was pigmented. In this case, the wild 30°C type bacteria grown in liquid culture had an $A_{534}/OD_{600}$ value of $0.003 \pm 0.001$, the

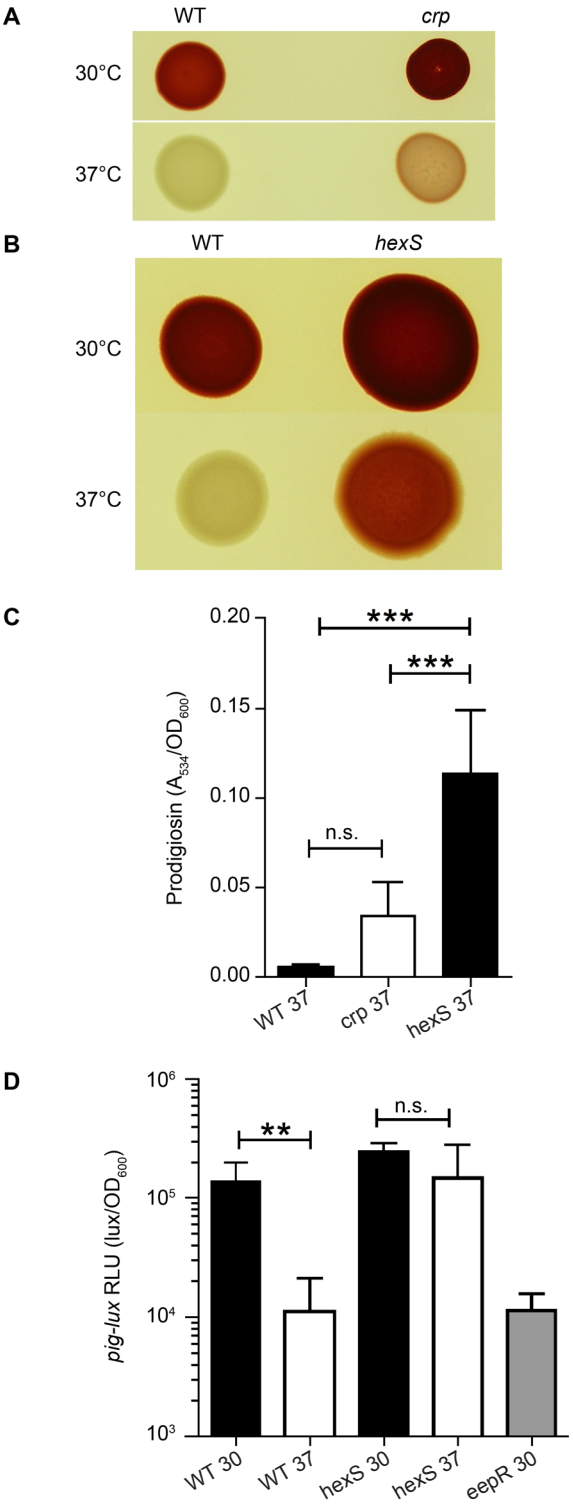


Fig. 3. The *hexS* gene contributes to pigment suppression at 37°C.

A and B. Pigment production at 30°C and 37°C after 18 h of growth at 30°C. The *hexS* mutant retains the ability to produce pigment at 37°C. The increased colony size of the *hexS* mutant reflects elevated serrawettin production. Images depict macrocolonies resulting from spotting broth from liquid culture onto an LB agar plate. C. Prodigiosin measured from macrocolonies grown at 37°C for 24 hours, normalized by $OD_{600}$. D. A plasmid-borne *luxCDABE* reporter for *pig* transcription was used to measure the importance of the *hexS* gene in temperature regulation. Unlike the WT, the *hexS* mutant was largely unaffected by growth at 37°C. The *eepR* mutant served as a control for low levels of *pig* transcription. Asterisks indicate significant differences by ANOVA with Tukey's post-test (** – $p < 0.01$, *** – $p < 0.001$, n = 8). n.s. indicates not significant.

*crp* mutant generated 0.004 ± 0.002, and the *hexS* produced significantly more 0.012 ± 0.004 (n = 6, $p < 0.001$, ANOVA with Tukey's post-test). These results indicate that HexS has a prominent role in temperature-dependent pigment inhibition. Consistently, the *hexS* gene was found in a genetic screen in which it produced slightly pigmented colonies at 37°C in *S. marcescens* strain 274 (Tanikawa et al. 2006).

To further analyze the impact of HexS on *pig* transcription, a plasmid-borne *pig-lux* reporter was generated and used to test the prediction that *pig* transcription is increased at 37°C in the *hexS* mutant relative to the wild type. While the plasmid-borne reporter has a much higher background than the chromosomal reporters (Fig. 3D versus Fig. 1B) it was much easier to move the reporter plasmid to different strain backgrounds. A greater than10-fold reduction in RLU measured from the *pig-lux* reporter was measured from the wild type grown at 37°C compared to 30°C. Furthermore, expression from the wild type at 37°C was indistinguishable from *pig-lux* measured from an *eepR* mutant grown at 30°C (Fig. 3D). This mutant is unable to make pigment because it lacks, EepR, a positive regulator of *pig* transcription. These data validated the reporter construct, so it was moved into the *hexS* mutant. There was no significant difference in *pig-lux* measured from the *hexS* mutant at 30°C versus 37°C ($p > 0.05$ ANOVA with Tukey's post-test), although there was a slight downward trend.

**Pigment thermoregulation is EepR and PigP independent.** In previous studies, it was determined that the transcription factor genes *eepR* and *pigP* were more highly expressed in *crp* and *hexS* mutants and that EepR and PigP were positive regulators of *pig* transcription in *S. marcescens* and another *Serratia* species (Fineran et al. 2005; Shanks et al. 2013; Stella et al. 2015; Shanks et al. 2017a). A model depicting the interaction between these transcription factors and the *pig* operon is shown in Fig. 4. Based on these data, we predicted that the increased expression of *eepR* and *pigP* in the *crp* and *hexS* mutants could be responsible for the increased pigmentation at 37°C. The wild-type strain with either a vector or wild-type *eepR* and *pigP* on a plasmid was grown at 30°C and 37°C, and while more pigmentation was observed at 30°C with the *eepR* and *pigP* plasmids, no pigmentation was observed at 37°C (Fig. 5). These plasmids were previously shown to complement *eepR* and *pigP* mutant phenotypes and are under control of the *E. coli lac* promoter (Shanks et al. 2013). We confirmed that the *lac* promoter is highly expressed in *S. marcescens* at both 30°C and 37°C using a transcriptional *hcred* fluorescent reporter-fusion. Although fluorescence was 2.7-fold higher at 30°C than 37°C (10745 ± 1019 RFU at 30°C versus 3997 ± 947 RFU at 37°C), at both temperatures fluorescence was much higher than background levels of ~10 RFU at both temperatures. Together these control experiments suggest that the plasmids are functional and expressing the genes at both temperatures. Moreover, data presented here suggest that thermoregulation of pigmentation is largely independent of EepR and PigP and is partially due to direct repression of *pig* by HexS at 37°C.

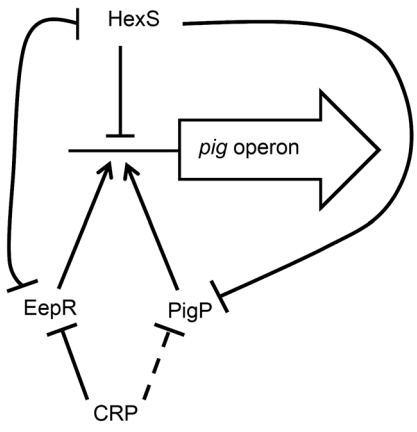


Fig. 4. Model genetic circuit used in this study. In this regulatory circuit, the role of several transcriptional regulators in control of the *pig* operon promoter and each other is depicted. Arrows indicate positive regulation and bars indicate negative regulation of transcription. All interactions have been shown to be direct except CRP inhibition of *pigP* expression (dotted line). Evidence from this study suggests that HexS inhibition of *pig* operon expression is a major reason for lack of *S. marcescens* pigmentation at 37°C.

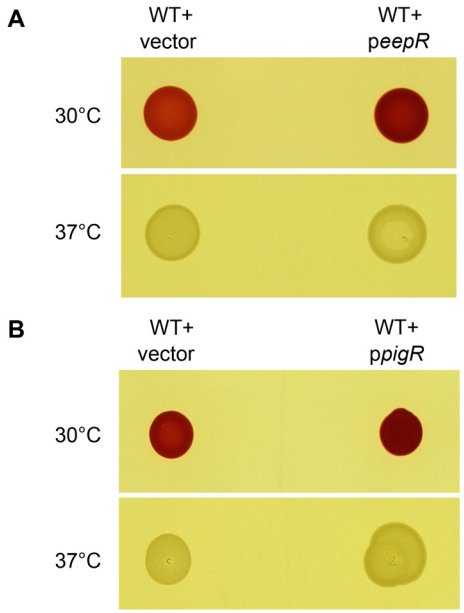


Fig. 5. Multicopy expression of *eepR* and *pigP* does not restore pigmentation at 37°C. Bacteria were plated on LB medium, incubated at 30 and 37°C for 18 hours and photographed. Multicopy expression of *eepR* (A) and *pigP* (B), increased pigmentation at 30°C, but not 37°C. Vector – pMQ132; p*eepR* – pMQ364; p*pigP* – pMQ221. Images show macrocolonies resulting from spotting broth from liquid culture onto LB agar plates.

## Discussion

The results of our study indicate that thermoregulation predominantly occurs at the transcriptional level. Our study: 1) correlated the nonpermissive temperature with a lack of *pig* transcription, and 2) demonstrated that artificial induction of *pig* operon transcription was sufficient for pigmentation at 37°C. This latter result indicated that the prodigiosin biosynthetic genes were translated and function at the nonpermissive temperature and that the chemical substrates needed to generate prodigiosin were present in the cell at the non-permissive temperature. Whereas post-transcriptional thermoregulation may be occurring, it was below the level of detection of the assays used in this study. Therefore, the findings in this study support the conclusion that thermoregulation of prodigiosin biosynthesis by *S. marcescens* occurs predominantly at the transcriptional level.

There are a large number of other stimuli that influence prodigiosin production at the transcriptional level, such as sugars, phosphate, ATP, cyclic nucleotides, indole, and quorum sensing molecules in *S. marcescens* and other *Serratia* species, presumably through the action of transcription factors that respond to the stimuli (Williamson et al. 2006; Haddix et al. 2008; Fender et al. 2012; Hidalgo-Romano et al. 2014; Haddix and Shanks 2018). It is possible that altered concentrations of one or more of these factors at 37°C is responsible for the pigment inhibition effect. Negative regulators of *S. marcescens* prodigiosin production include CRP (Kalivoda et al. 2010; Stella and Shanks 2014), HexS (Tanikawa et al. 2006; Stella et al. 2012), and RssAB (Horng et al. 2010). Previously, Horng and colleagues (2010) tested whether mutation of the *rssBA* genes would restore pigmentation at 37°C, and found that the CH-1 strain of *S. marcescens* with mutation of *rssA* or both *rssA* and *rssB* did not produce prodigiosin at 37°C. The authors concluded that prodigiosin production is inhibited by an unknown mechanism independent of the RssB/A two-component system (Horng et al. 2010). Here, we found that mutation of *crp* allowed production of faintly red colonies at 37°C, but liquid cultures were not red at 37°C. This may be due to a number of different variables between growth in liquid and on plates such as oxygen levels, as oxygen is necessary for pigment production (Heinemann et al. 1970). A similar pattern was observed at 30°C with a *pigP* mutant of strain PIC3611 and *gumB* and *eepR* mutants of the strain K904 background, all of which produced pink colonies on plates, but no detectable pigmentation in aerated liquid cultures (Shanks et al. 2013; Stella et al. 2015; Stella et al. 2018). Another example is evident with the wild-type strain grown at 30°C on LB agar plates becomes bright red (Fig. 1A) whereas in LB broth, the bacteria are clearly less pigmented (Fig. 2A). Evidence suggests that CRP does not directly bind to the pigment biosynthetic operon promoter (Kalivoda et al. 2010), implying that one or more of the many genes regulated by CRP, in turn, alters *pig* operon expression at 37°C. The CRP protein is implicated in heat shock responses in *E. coli* and other bacteria, indicating that a *crp* mutation can impact bacterial temperature responses (Nagai et al. 1990; Choudhary et al. 2014). Here, we found a relatively minor role for CRP, with some pigment being observed on agar plates in the *crp* mutant at 37°C.

The *hexS* gene of *S. marcescens* was originally isolated by Tanikawa and colleagues (2006) in a screen for mutations, in strain 274, that allowed prodigiosin and serrawettin W1 (also known as serratamolide) production at 37°C. The *hexS* mutant produced a faintly red colony at 37°C and overproduced progidiosin at 30°C. This result was different from our study where the *hexS* mutant produced a robustly red colony at 37°C on the same medium over a similar time frame suggesting that there may be some differences between pigment regulation between strains, as has been shown for cyclic-AMP regulation of prodigiosion (Stella and Shanks 2014). Nevertheless, these studies confirm that HexS is a key transcription factor in the inhibition of pigment biosynthesis at 37°C.

Given the potential of prodigiosin and similar compounds as antimicrobial, antitumor, and anti-inflammatory therapeutics (Perez-Tomas et al. 2003; Williamson et al. 2006; Perez-Tomas and Vinas 2010), understanding regulation of the natural biosynthesis of these compounds can inform their industrial production. This study provides evidence that barriers to prodigiosin production at 37°C can be overcome at the transcriptional level, and that mutations such as in *hexS* can be used to increase prodigiosin production, and provides insight into the basic biological question of secondary metabolite thermoregulation by *S. marcescens*.

**ORCID**
Robert M.Q. Shanks 0000-0002-7699-5396

**Acknowledgments**

The authors would like to thank Mohammed AlHigaylan and Kimberly Brothers for critical reading of the manuscript. This work was supported by NIH Core Vision Research grant EY08098, NIH grant AI085570, unrestricted funds from the Research to Prevent Blindness, and the Eye and Ear Foundation of Pittsburgh.

**Conflict of interest**

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

## Literature


**Barriuso J, Hogan DA, Keshavarz T, Martínez MJ.** Role of quorum sensing and chemical communication in fungal biotechnology and pathogenesis. FEMS Microbiol Rev. 2018;42(5):627–638. doi:10.1093/femsre/fuy022 Medline

**Benedetti IM, de Lorenzo V, Silva-Rocha R.** Quantitative, non-disruptive monitoring of transcription in single cells with a broad-host range GFP-*luxCDABE* dual reporter system. PLoS One. 2012;7(12):e52000. doi:10.1371/journal.pone.0052000 Medline

**Bertani G.** Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli.* J Bacteriol. 1951;62(3):293–300. Medline

**Blizzard JL, Peterson GE.** Selective inhibition of proline-induced pigmentation in washed cells of *Serratia marcescens.* J Bacteriol. 1963;85:1136–1140. Medline

**Cheng MF, Lin CS, Chen YH, Sung PJ, Lin SR, Tong YW, Weng CF.** Inhibitory growth of oral squamous cell carcinoma cancer via bacterial prodigiosin. Mar Drugs. 2017;15(7):224. doi:10.3390/md15070224 Medline

**Chiang SL, Rubin EJ.** Construction of a mariner-based transposon for epitope-tagging and genomic targeting. Gene. 2002;296 (1–2):179–185. doi:10.1016/S0378-1119(02)00856-9 Medline

**Choi SY, Im H, Mitchell RJ.** Violacein and bacterial predation: promising alternatives for priority multidrug resistant human pathogens. Future Microbiol. 2017;12(10):835–838. doi:10.2217/fmb-2017-0090 Medline

**Choudhary E, Bishai W, Agarwal N.** Expression of a subset of heat stress induced genes of *Mycobacterium tuberculosis* is regulated by 3',5'-cyclic AMP. PLoS One. 2014;9(2):e89759. doi:10.1371/journal.pone.0089759 Medline

**Danevčič T, Borić Vezjak M, Tabor M, Zorec M, Stopar D.** Prodigiosin induces autolysins in actively grown *Bacillus subtilis* cells. Front Microbiol. 2016a;7:27. doi:10.3389/fmicb.2016.00027 Medline

**Danevčič T, Borić Vezjak M, Zorec M, Stopar D.** Prodigiosin – A multifaceted *Escherichia coli* antimicrobial agent. PLoS One. 2016b;11(9):e0162412. doi:10.1371/journal.pone.0162412 Medline

**Daniels R, Vanderleyden J, Michiels J.** Quorum sensing and swarming migration in bacteria. FEMS Microbiol Rev. 2004;28(3):261–289. doi:10.1016/j.femsre.2003.09.004 Medline

**Demain AL, Fang A.** The natural functions of secondary metabolites. Adv Biochem Eng Biotechnol. 2000;69:1–39. doi:10.1007/3-540-44964-7_1 Medline

**Fender JE, Bender CM, Stella NA, Lahr RM, Kalivoda EJ, Shanks RMQ.** *Serratia marcescens* quinoprotein glucose dehydrogenase activity mediates medium acidification and inhibition of prodigiosin production by glucose. Appl Environ Microbiol. 2012;78(17):6225–6235. doi:10.1128/AEM.01778-12 Medline

**Fineran PC, Slater H, Everson L, Hughes K, Salmond GPC.** Biosynthesis of tripyrrole and β-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(6):1495–1517. doi:10.1111/j.1365-2958.2005.04660.x Medline

**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(22):7453–7463. doi:10.1128/JB.00909-08 Medline

**Haddix PL, Shanks RMQ.** Prodigiosin pigment of *Serratia marcescens* is associated with increased biomass production. Arch Microbiol. 2018;200(7):989–999. doi:10.1007/s00203-018-1508-0 Medline

**Hage-Hülsmann J, Grünberger A, Thies S, Santiago-Schübel B, Klein AS, Pietruszka J, Binder D, Hilgers F, Domröse A, Drepper T, et al.** Natural biocide cocktails: combinatorial antibiotic effects of prodigiosin and biosurfactants. PLoS One. 2018;13(7):e0200940. doi:10.1371/journal.pone.0200940 Medline

**Heinemann B, Howard AJ, Palocz HJ.** Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by *Serratia marcescens.* Appl Microbiol. 1970;19(5):800–804. Medline

**Hidalgo-Romano B, Gollihar J, Brown SA, Whiteley M, Valenzuela E Jr, Kaplan HB, Wood TK, McLean RJC.** Indole inhibition of N-acylated homoserine lactone-mediated quorum signalling is widespread in Gram-negative bacteria. Microbiology. 2014;160(Pt_11):2464–2473. doi:10.1099/mic.0.081729-0 Medline

**Hoefler BC, Stubbendieck RM, Josyula NK, Moisan SM, Schulze EM, Straight PD.** A Link between linearmycin biosynthesis and extracellular vesicle genesis connects specialized metabolism and bacterial membrane physiology. Cell Chem Biol. 2017;24: 1238–1249.e7. doi:10.1016/j.chembiol.2017.08.008 Medline

**Horng YT, Chang KC, Liu YN, Lai HC, Soo PC.** The RssB/RssA two-component system regulates biosynthesis of the tripyrrole antibiotic, prodigiosin, in *Serratia marcescens.* Int J Med Microbiol. 2010;300(5):304–312. doi:10.1016/j.ijmm.2010.01.003 Medline

**Im H, Choi SY, Son S, Mitchell RJ.** Combined application of bacterial predation and violacein to kill polymicrobial pathogenic communities. Sci Rep. 2017;7(1):14415. doi:10.1038/s41598-017-14567-7 Medline

**Kadouri DE, Shanks RMQ.** Identification of a methicillin-resistant *Staphylococcus aureus* inhibitory compound isolated from *Serratia marcescens.* Res Microbiol. 2013;164(8):821–826. doi:10.1016/j.resmic.2013.06.002 Medline

**Kalivoda EJ, Stella NA, Aston MA, Fender JE, Thompson PP, Kowalski RP, Shanks RMQ.** Cyclic AMP negatively regulates prodigiosin production by *Serratia marcescens.* Res Microbiol. 2010;161(2):158–167. doi:10.1016/j.resmic.2009.12.004 Medline

**Khan A, Singh P, Srivastava A.** Synthesis, nature and utility of universal iron chelator – Siderophore: A review. Microbiol Res. 2018;212-213:103–111. doi:10.1016/j.micres.2017.10.012 Medline

**Klein AS, Brass HUC, Klebl DP, Classen T, Loeschcke A, Drepper T, Sievers S, Jaeger KE, Pietruszka J.** Preparation of cyclic prodiginines by mutasynthesis in *Pseudomonas putida* KT2440. Chembiochem. 2018;19:1445–1552. Medline

**Klein AS, Domröse A, Bongen P, Brass HUC, Classen T, Loeschcke A, Drepper T, Laraia L, Sievers S, Jaeger KE, et al.** New prodigiosin derivatives obtained by mutasynthesis in *Pseudomonas putida.* ACS Synth Biol. 2017;6(9):1757–1765. doi:10.1021/acssynbio.7b00099 Medline

**Lin SR, Fu YS, Tsai MJ, Cheng H, Weng CF.** Natural compounds from herbs that can potentially execute as autophagy inducers for cancer therapy. Int J Mol Sci. 2017;18(7):1412. doi:10.3390/ijms18071412 Medline

**Nagai H, Yano R, Erickson JW, Yura T.** Transcriptional regulation of the heat shock regulatory gene *rpoH* in *Escherichia coli*: involvement of a novel catabolite-sensitive promoter. J Bacteriol. 1990; 172(5):2710–2715. doi:10.1128/jb.172.5.2710-2715.1990 Medline

**Pérez-Tomás R, Montaner B, Llagostera E, Soto-Cerrato V.** The prodigiosins, proapoptotic drugs with anticancer properties. Biochem Pharmacol. 2003;66(8):1447–1452. doi:10.1016/S0006-2952(03)00496-9 Medline

**Pérez-Tomás R, Viñas M.** New insights on the antitumoral properties of prodiginines. Curr Med Chem. 2010;17(21):2222–2231. doi:10.2174/092986710791331103 Medline

**Shanks RMQ, 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(7):5027–5036. doi:10.1128/AEM.00682-06 Medline

**Shanks RMQ, Kadouri DE, MacEachran DP, O'Toole GA.** New yeast recombineering tools for bacteria. Plasmid. 2009;62(2):88–97. doi:10.1016/j.plasmid.2009.05.002 Medline

**Shanks RMQ, Lahr RM, Stella NA, Arena KE, Brothers KM, Kwak DH, Liu X, Kalivoda EJ.** A *Serratia marcescens* PigP homolog controls prodigiosin biosynthesis, swarming motility and hemolysis and is regulated by cAMP-CRP and HexS. PLoS One. 2013;8(3):e57634. doi:10.1371/journal.pone.0057634 Medline

**Shanks RMQ, Stella NA, Lahr RM, Aston MA, Brothers KM, Callaghan JD, Sigindere C, Liu X.** Suppressor analysis of *eepR* mutant defects reveals coordinate regulation of secondary metabolites and serralysin biosynthesis by EepR and HexS. Microbiology. 2017;163(2):280–288. doi:10.1099/mic.0.000422 Medline

**Slater H, Crow M, Everson L, Salmond GPC.** Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol Microbiol. 2003;47(2):303–320. doi:10.1046/j.1365-2958.2003.03295.x Medline

**Stella NA, Brothers KM, Callaghan JD, Passerini AM, Sigindere C, Hill PJ, Liu X, Wozniak DJ, Shanks RMQ.** An IgaA/UmoB-family protein from *Serratia marcescens* regulates motility, capsular polysaccharide, and secondary metabolite production. Appl Environ Microbiol. 2018;84(6):e02575-17. doi:10.1128/AEM.02575-17 Medline

**Stella NA, Fender JE, Lahr RM, Kalivoda EJ, Shanks RM.** The LysR transcription factor, HexS, is required for glucose inhibition of prodigiosin production by *Serratia marcescens.* Adv Microbiol. 2012;2(4):511–517. doi:10.4236/aim.2012.24065 Medline

**Stella NA, Lahr RM, Brothers KM, Kalivoda EJ, Hunt KM, Kwak DH, Liu X, Shanks RMQ.** *Serratia marcescens* cyclic AMP-receptor protein controls transcription of EepR, a novel regulator of antimicrobial secondary metabolites. J Bacteriol. 2015;197(15):2468–2478. doi:10.1128/JB.00136-15 Medline

**Stella NA, Shanks RMQ.** Cyclic-AMP inhibition of fimbriae and prodigiosin production by *Serratia marcescens* is strain-dependent. Arch Microbiol. 2014;196(5):323–330. doi:10.1007/s00203-014-0970-6 Medline

**Tanaka Y, Yuasa J, Baba M, Tanikawa T, Nakagawa Y, Matsuyama T.** Temperature-dependent bacteriostatic activity of *Serratia marcescens.* Microbes Environ. 2004;19(3):236–240. doi:10.1264/jsme2.19.236

**Tanikawa T, Nakagawa Y, Matsuyama T.** Transcriptional downregulator hexS controlling prodigiosin and serrawettin W1 biosynthesis in *Serratia marcescens*. Microbiol Immunol. 2006;50(8):587–596. doi:10.1111/j.1348-0421.2006.tb03833.x Medline

**Vining LC.** Functions of secondary metabolites. Annu Rev Microbiol. 1990;44(1):395–427. doi:10.1146/annurev.mi.44.100190.002143 Medline

**Waters CM, Bassler BL.** Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 2005;21(1):319–346. doi:10.1146/annurev.cellbio.21.012704.131001 Medline

**Williams RP, Goldschmidt ME, Gott CL.** Inhibition by temperature of the terminal step in biosynthesis of prodigiosin. Biochem Biophys Res Commun. 1965;19(2):177–181. doi:10.1016/0006-291X(65)90500-0 Medline

**Williamson NR, Fineran PC, Leeper FJ, Salmond GPC.** The biosynthesis and regulation of bacterial prodiginines. Nat Rev Microbiol. 2006;4(12):887–899. doi:10.1038/nrmicro1531 Medline