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The LysR Transcription Factor, HexS, Is Required for Glucose Inhibition of Prodigiosin Production by *Serratia marcescens*

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ABSTRACT

Generation of many useful microbe-derived secondary metabolites, including the red pigment prodigiosin of the bacterium *Serratia marcescens*, is inhibited by glucose. In a previous report, a genetic approach was used to determine that glucose dehydrogenase activity (GDH) is required for inhibiting prodigiosin production and transcription of the prodigiosin biosynthetic operon (*pigA-N*). However, the transcription factor(s) that regulate this process were not characterized. Here we tested the hypothesis that HexS, a LysR-family transcription factor similar to LrhA of *Escherichia coli*, is required for inhibition of prodigiosin by growth in glucose. We observed that mutation of the *hexS* gene in *S. marcescens* allowed the precocious production of prodigiosin in glucose-rich medium conditions that completely inhibited prodigiosin production by the wild type. Unlike previously described mutants able to generate prodigiosin in glucose-rich medium, *hexS* mutants exhibited GDH activity and medium acidification similar to the wild type. Glucose inhibition of *pigA* expression was shown to be dependent upon HexS, suggesting that HexS is a key transcription factor in secondary metabolite regulation in response to medium pH. These data give insight into the prodigiosin regulatory pathway and could be used to enhance the production of secondary metabolites.

Keywords: Pigment; Antibiotic; Transcription Factor; Secondary Metabolite

1. Introduction

The production of many secondary metabolites by microorganisms, such as antibiotics, is inhibited by growth in glucose-rich medium [1,2]. This is unfortunate as glucose is an ideal carbon source for growth of many microorganisms. The Gram-negative bacterium *Serratia marcescens* is a model to study secondary metabolites because it generates the tractable tripyrrole pigment, prodigiosin [3]. Glucose inhibits prodigiosin production by *S. marcescens* [4] due to acidification of the medium [1,2], but the mechanism of this inhibition is incompletely understood.

Fender, *et al.*, recently identified genes required for glucose dehydrogenase (GDH)-dependent inhibition of prodigiosin by medium acidification [5]. It was demonstrated that GDH activity in glucose rich medium is necessary for the rapid culture pH reduction, resulting in a complete inhibition of prodigiosin production. Expression of the prodigiosin biosynthetic operon, *pigA-N* [6,7] was inhibited by glucose-induced acidification, suggesting that prodigiosin inhibition is largely controlled at the transcriptional level. The transcription factors responsible for this regulation are unknown. However, a clue to the identification of the unknown factor(s) was revealed in a recent genetic screen [5]. Fender and colleagues observed that mutation in the gene that codes for the LysR-family transcription factor, HexS, allowed the bacterium to generate prodigiosin in glucose-rich medium in a *crp* mutant strain. However, the impact of mutation of *hexS* on glucose inhibition of secondary metabolites was not characterized and the phenotype was not verified in a strain with a functional CRP protein.

The goal of this study was to determine whether the transcription factor HexS is necessary for glucose-mediated inhibition of secondary metabolite production, using prodigiosin as a model secondary metabolite.

HexS was previously found in a genetic screen for genes that control production of the secondary metabolites, serratamolide and prodigiosin, and was also shown to directly regulate the prodigiosin biosynthetic operon [8]. The only other report featuring HexS indicates that it

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negatively regulates production of the antimicrobial biosurfactant serratamolide [9]. HexS is highly similar LrhA of *Escherichia coli* [10] and is well conserved among the Enterobacteriaceae. HexS is also similar to HexA proteins from *Pectobacterium* and *Photorhabdus* species, and PigU from *Serratia* sp. ATCC 39006, which are regulators of secreted enzymes and metabolites [11-14]. LrhA regulates a number of processes including motility, chemotaxis, and aspects of stationary phase physiology partially through indirect control of sigma factor S [10].

This brief report provides genetic and biochemical evidence that HexS is necessary for glucose to inhibit prodigiosin production and transcription from the prodigiosin biosynthetic operon by *S. marcescens*.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

All microorganisms are listed in **Table 1** and were grown in lysogeny broth (LB) [15,16] with or without D-glucose (110 mM), with the exception of *Saccharomyces cerevisiae* which was grown with SC-uracil or YPD broth [17]. Cultures were grown in 5 mL aliquots of medium and rotated at high speed on a TC-7 tissue culture rotor (New Brunswick Scientific). Antibiotics used were tetracycline (10 μg/mL), kanamycin (100 μg/mL) and gentamicin (10 μg/mL). Conjugations were performed using *Escherichia coli* donor strain S17-1 as previously described [5]. Time course experiments were performed as previously described [5].

2.2. Cloning and Genetic Manipulations

The *hexS* open reading frame (ORF) was cloned using primers gaattgtgagcggataacaatttcacacaggaaacagctATGACAACTGCAAATCGTCCG (uppercase describes priming DNA, and lowercase represents recombination targeting sequence) and gttttatcagaccgcttctgcgttctgatttagTTATTCTTCTTCGTCCACCAGGCTGGC using yeast *in vivo* cloning [18] into integrative plasmid pMQ236 [19] to generate pMQ294. The *hexS*-Δ1 allele was made by

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
S. cerevisiae		
InvSc1	Diploid uracil auxotroph	Invitrogen
Bacterial strains		
S17-1 λpir	*Escherichia coli* strain used for conjugation	[22]
EC100D	*E. coli* strain used for cloning, *pir*-116	Epicentre
ER2566	*E. coli* strain used for protein expression	New England Biolabs
CMS376	wild-type *Serratia marcescens*, PIC strain number 3611	Presque Isle Cultures
CMS1779	*hexS*::pStvz3 in WT (CMS376), *hexS*-1	This study
CMS2210	Δ*hexS*, (*hexS*-Δ1 allele) in CMS376	This study
CMS3125	CMS2210 + pMQ294 *hexS* complementation strain	This study
CMS3142	*nuoC*::pStvZ3 in CMS376	This study
CMS1317	*alaT*::pMQ118 in CMS376	This study
Plasmids		
pMal-C2	Maltose binding protein fusion construct	New England Biolabs
pStvZ3	*ori*R6K *lacZ nptII* promoter probe	[20]
pMQ118	suicide vector *nptII*, *rpsL*, *oriT*, *URA*3, *CEN6/ARSH4*	[19]
pMQ197	pMQ118 with internal fragment of *alaT*	This study
pMQ236	allelic replacement vector, pMQ118 with I-SceI site	[19]
pMQ240	I-SceI-expression plasmid, *aacC*1, *ori*pSC101^{ts}	[19]
pMQ268	pStvZ3 + *pigA* internal fragment	[20]
pMQ294	pMQ236 with *hexS* open reading frame	This study
pMQ296	pMQ294 with *hexS*-Δ1 mutant allele	This study
pMQ342	pStvZ3 with internal fragment of *nuoC*	This study
pMQ402	pMAL-C2 + *hexS* (MBP-HexS fusion construct)	This study

digesting pMQ294 with SacII, which cuts at a single site in the *hexS* ORF. The SacII digested plasmid was treated with the End-IT kit (Epicentre) to generate blunt ends and the plasmid was recircularized using T4 DNA ligase (New England Biolabs). The resulting plasmid, pMQ296, contained a *hexS* gene with a one base pair deletion (base pair 670 out of a total of 945 for the gene) creating a frame shift mutation and truncation.

For the insertional mutagenesis of *alaT*, *hexS* and *nuoC*, internal fragments were amplified using primers tgggtaacgccagggttttcccagtcacgacgttgtaCCGTTACGACATGCATTTGCACC and gataacaatttcacacaggaaacagctatgaccatgatGGACGATCAGCTCAGAGACGC for *alaT*, gttgggtaacgccagggttttcccagtcacgacgttgtaCGCGCACGTCAATGGCCAGC and taacaatttcacacaggaaacagctatgaccatgatCCTCGATCTGCTAAGAACCTTTG for *hexS*, and gacgttgtaaaacgacgggatctatcatcgtggatccTGGTGATAACCGATGTCCGGCAC and ctagagcggtttcccgactggaaagcgggcagtgagcgcCGAACTGCGCAACCGTTTTGG for *nuoC*. The amplicons were cloned into pMQ118 or pStvZ3 [19,20] and the resulting plasmids were introduced into *S. marcescens* by conjugation and selection for kanamycin resistance. The resulting strains have the plasmid inserted before the midpoint of each gene. Mutations were verified by PCR and mutant phenotype.

For purification of recombinant HexS, a N-terminal maltose binding protein (MBP) fusion was created in which the entire *hexS* open reading frame was fused to the *malE* gene using pMal-C2 (New England Biolabs). The open reading frame was amplified with primers that incorporated EcoR1 and PstI sites for subsequent digestion and T4-ligase based ligation into the pMal-C2 plasmid polylinker. The resulting construct was verified by sequencing across the *malE-hexS* junction.

Allelic replacement of *hexS* with deletion alleles was performed using pMQ296, respectively, with previously described protocols [19], and was verified by PCR, sequencing, and phenotypic analysis. Briefly, pMQ296 was introduced into the recipient strain by conjugation and selection for kanamycin resistance (100 μg/mL) to generate a merodiploid. The I-SceI expressing plasmid pMQ240 was introduced into the merodiploid by conjugation with selection for gentamicin (10 μg/mL). The merodiploid strain with pMQ240 was grown in LB with gentamicin and streaked to single colonies on LB agar plates. Colonies were tested individually for loss of kanamycin resistance, and kanamycin susceptible strains were assessed for deletion of *pigA* or *hexS* using PCR. The *pigA*::*lacZ* construct was generated using pMQ268 as previously described [20], where the pStvZ3 plasmid bearing an internal fragment of *pigA* was introduced into recipient strains by conjugation and selection for kanamycin (100 μg/mL).

2.3. Enzymatic Assays and Electrophoretic Mobility Shift Assay (EMSA)

Measurement of *β*-galactosidase activity was performed as previously described using 200 μg of cell lysate from stationary phase cultures that had grown for 20 hours at 30˚C using o-nitrophenyl *β*-D-galactopyranoside (ONPG) as a chromogenic substrate [20].

D-gluconic acid concentration was measured using an enzymatic D-gluconic acid detection kit (Megazyme, product K-GATE) as described by the manufacturer, with modifications that were previously described [5].

Glucose dehydrogenase activity (GDH) was measured using a chromogenic reaction based on the method of Matsushita, *et al.* [21] using 2,6-Dichlorophenol-Indophenol (Sigma, D-1878) and 13 mM phenazine methosulfate (Sigma, P-9625) as substrates as previously described [5].

A maltose binding protein (MBP) fusion of HexS and the MBP alone were purified using the manufacturers specifications (pMal Protein Fusion and Purification System, New England Biolabs). EMSA reactions were performed as previously described [20] using a comercial EMSA kit (Lightshift Chemiluminescent EMSA kit, Pierce, Rockford IL). Briefly, *pigA* promoter DNA was amplified using biotinylated (or non-biotinylated) oligonucleotide primer 5'-ggggtacccttcacctgcaaagtattcatc-3' and non-biotinylated primer cggaattcagtatcgagcgcattcatgcc. The resulting amplicon was gel purified and used at 2 ng per binding reaction following the manufacturers specifications. A 10 μL aliquot of the reaction was separated in a TBE PAGE gel (Pierce), transferred to a nylon membrane, and examined through chemiluminescence according to the manufacturers specifications. MBP and MBP-HexS were used at 25 - 30 μg per reaction, and unlabelled promoter DNA was amplified with unlabelled versions of the above listed primers and the amplicon was used at 500 ng per binding reaction where specified. The EMSA reaction was performed three times with consistent results.

3. Results and Discussion

3.1. Mutation of *hexS* Allows Prodigiosin Production in Glucose Rich Medium

In a recently reported genetic screen, we identified mutants that produced prodigiosin on LB glucose-supplemented agar plates (LBG) unlike the parental strain that was pigmentless on LBG [5]. One mutant strain that was pigmented on LBG agar had a transposon insertion in the gene for the HexS prodigiosin-regulatory transcription factor protein. The importance of HexS in glucose inhibition of prodigiosin by glucose was not verified in that study. Furthermore the previous study was performed using a *crp* mutant rather than a wild-type strain. The

goal of this brief study was to determine whether HexS is necessary to inhibit *S. marcescens* prodigiosin production in glucose rich medium.

To characterize the effect of the *hexS* mutant phenotype in a wild-type (WT strain CMS376) background, we generated a deletion allele *hexS*-Δ1, a directed *hexS* null-mutant allele to replace the chromosomal *hexS* gene in the WT (CMS376) strain, generating strain CMS2210, referred to here as Δ*hexS*. This mutant strain exhibits all of the characteristics of a *hexS* null mutant strain, such as elevated prodigiosin production and serratamolide production (data not shown). The Δ*hexS* (CMS2210) mutant and WT (CMS376) grew at the same rate in LB medium (**Figure 1(a)**). Unlike its growth-inhibitory effect upon the WT (CMS376), glucose did not inhibit culture density of the Δ*hexS* (CMS2210) mutant (**Figure 1(a)**). Furthermore, the Δ*hexS* (CMS2210) strain produced prodigiosin when grown with glucose unlike the WT (CMS376) (**Figure 1(b)**).

Previously described mutants able to produce pigment in LB medium supplemented with glucose (LBG) had mutations in genes necessary for glucose dehydrogenase activity (GDH), failed to achieve WT levels of medium acidification, and medium acidification was necessary for glucose to prevent prodigiosin production [5]. To test the prediction that *hexS* mutants are also able to generate pigment in LBG because they fail to acidify the medium, we measured the impact of *hexS* mutation upon medium acidification. No difference was observed between WT (CMS376) and the Δ*hexS* (CMS2210) mutant with regards to pH in LB (**Figure 1(c)**). In LBG, the Δ*hexS* (CMS2210) mutant differed from the WT (CMS376). Up to hour 6, both the WT (CMS376) and *hexS* mutant (CMS2210) experienced a sharp drop in pH, but after 6 hours the WT (CMS376) culture continued to decrease below pH 4, whereas the Δ*hexS* (CMS2210) culture reproducibly stabilized close to pH 5 and then increased to neutrality by t = 24 hours (**Figure 1(c)**). This result is in stark contrast to all other reported pigment positive mutants that did not support a glucose-induced pH reduction [5]. Interestingly, the HexS deficient strain produced prodigiosin in cultures with very low pH. These are important observations because it shows that prodigiosin can be produced in acidic medium, indicating that the enzymatic process of generating prodigiosin is not simply unable to occur at low pH, and implying that an active inhibition of prodigiosin production takes place in glucose-rich medium. The mechanism by which HexS mediates culture pH in LBG will be the subject of subsequent research.

3.2. Quinoprotein Glucose Dehydrogenase Protein Activity Is Altered in *hexS* Mutants

Previous work showed that reduced quinoprotein glucose dehydrogenase (GDH) activity, which converts glucose to gluconic acid, conferred the ability to produce prodigiosin in LBG [5]. Therefore, we tested whether the *hexS*

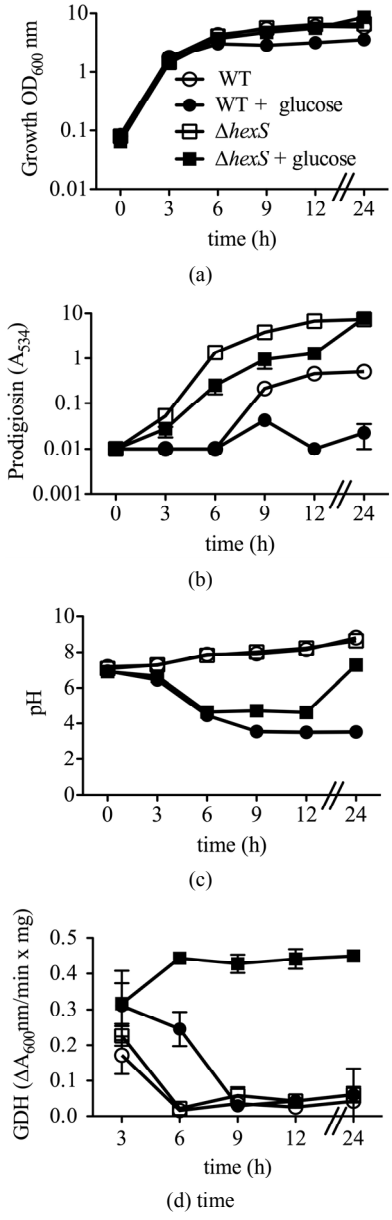

Figure 1. Growth, prodigiosin production, culture pH of WT and Δ*hexS* (CMS2210) mutant cells grown in LB and LBG. Genotype symbols are consistent for each panel. Cultures were grown in LB and in LBG (+glucose). The *hexS* mutant used was Δ*hexS* (CMS2210). (a) Culture growth as measured by turbidity (OD_{600} nm); (b) Extracted prodigiosin levels were measured from 1 mL of culture (A_{534} nm); (c) Culture pH was determined with a pH meter; (d) Glucose dehydrogenase activity measured from crude cellular extracts. Time course experiments were performed with 4 independent biological replicates per time point per condition, and performed three times with consistent results, one representative time course is shown. Error bars indicate one standard deviation.

mutant (CMS2210) would have reduced GDH activity. However, the Δ*hexS* (CMS2210) mutant surprisingly produced equivalent GDH activity to the WT (CMS376) when grown in LB, and higher levels than the WT (CMS376) at later time points when cultured in LBG (**Figure 1(d)**) suggesting the GDH activity in LBG is regulated by HexS.

As a second assay for GDH activity, we measured gluconic acid (GA) levels. GA in LBG was maximal for the wild type (CMA376) at 6 - 9 hours [5]. Unexpectedly, gluconic acid levels in the Δ*hexS* (CMS2210) mutant culture at six hours were significantly reduced compared to the WT (CMS376) (Student's T-test $p < 0.05$). In the aforementioned time course (**Figure 1**) gluconic acid was measured at 12.1 ± 1.9 mM from the Δ*hexS* (CMS2210) strain, compared 20.2 ± 2.4 mM for the WT (CMS376) at t = 6 hours. A similar trend was observed over the time course (data not shown). These data suggest that the reduced organic acid in Δ*hexS* (CMS2210) mutant cultures at late time points may account of the increased culture pH at late time points.

3.3. Complementation of the Δ*hexS* (CMS2210) Phenotype

To perform complementation analysis, we introduced a single WT copy of *hexS* into the Δ*hexS* (CMS2210) chromosome with pMQ294 (diagramed in **Figure 2(a)**) and restored the glucose sensitivity phenotype (**Figure 2(b)**). We measured elevated prodigiosin levels in both the *hexS* deletion strain and a *hexS*-1 (CMS1779) insertion mutant strain compared to WT (CMS376). As an additional control, *hexS* was mutated by plasmid insertion. This *hexS*-1 (CMS1779*)* strain served as a control to be an independent mutation to confirm that mutation of *hexS* conferred the observed pigment phenotypes, and to test whether insertion of a plasmid alone into a *hexS mutant* (CMS2210) strain would restore sensitivity to glucose. In LBG, the WT (CMS376) and Δ*hexS* (CMS2210) + pMQ294 results were not significantly different in prodigiosin production, whereas the *hexS* mutant's (CMS2210) pigment levels were significantly higher than either WT (CMS376) or Δ*hexS* (CMS2210) + pMQ294 (ANOVA, $p < 0.05$, **Figure 2**). To further ensure that the pigment phenotype of *hexS* mutants were not due to a polar effect on adjacent genes, mutations were introduced into the operons on either side of *hexS* on the chromosome. In one direction is a divergently transcribed *alaT* homolog greater than 650 base pairs away from the *hexS* gene, and on the opposite side of *hexS*, transcribed in the same orientation, is a predicted NADH-ubiquinone oxidoreducatase operon (*nuoA-N*), greater than 700 base pairs away from the *hexS* ORF. To further support that loss of the glucose inhibition of

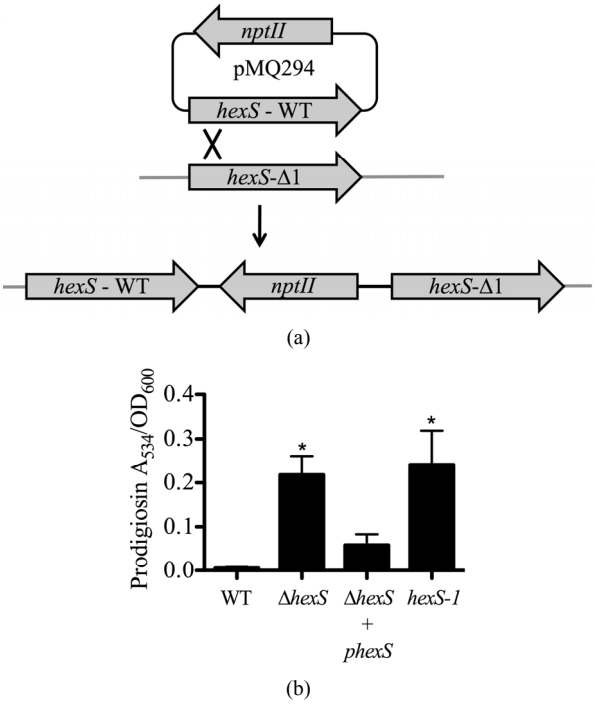

Figure 2. Complementation of the *hexS* mutant phenotype. (a) Complementation strategy through integrating a wild-type *hexS* gene on pMQ294 into a strain with a *hexS* deletion mutation (CMS3125). (b) Prodigiosin production in LBG shows that unlike the WT (CMS376), a *hexS* deletion mutant (Δ*hexS*, CMS2210) and a *hexS* insertion mutant (*hexS*-1, CMS1779) were able to generate prodigiosin. A wild-type copy of *hexS* on a plasmid (pMQ294) was inserted into the chromosome of the Δ*hexS* (CMS2210) strain and restored glucose sensitivity. Error bars indicate one standard deviation, and asterisks indicate significant differences from the WT (CMS376) ($p < 0.05$, ANOVA with Tukey's post-test).

prodigiosin phenotype of *hexS* mutants was not due to a polar effect on adjacent genes, *alaT* and *nuoC* were separately mutated in the WT (CMS376) strain. The resulting mutant strains did not exhibit the pigmented phenotype of the *hexS* insertion mutant in LBG (data not shown). Together, these data support that HexS is important for glucose inhibition of pigmentation.

3.4. HexS Is Necessary for Glucose-Inhibition of Transcription of the Prodigiosin Biosynthetic Operon

To test the hypothesis that HexS regulates the *pigA* promoter in LBG, we used a previously characterized chromosomal *pigA-lacZ* reporter [20]. Whereas *pigA-lacZ* associated *β*-galactosidase levels were severely reduced in the WT (CMS376) in LBG compared to LB medium, the Δ*hexS* (CMS2210) mutant exhibited similar expression levels regardless of glucose (**Figure 3(a)**). To ensure that this was a direct transcriptional regulation, we con

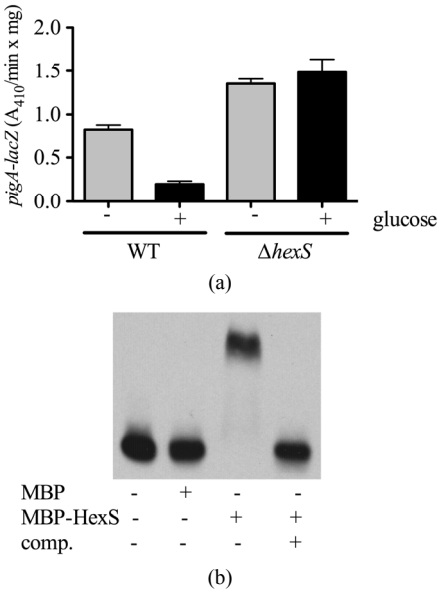

Figure 3. Glucose sensitivity of the prodigiosin biosynthetic operon is dependent upon HexS. (a) Expression from the *pigA* promoter was indirectly measured using a chromosomal *lacZ* transcriptional fusion. *β*-galactosidase activity was measured from cells grown to stationary phase (20 h) from culture grown in LB (−) or LBG (+) medium. Error bars indicate one standard deviation; (b) Representative EMSA showing MBP-HexS binding to the biotinylated *pigA* promoter. MBP = maltose binding protein, MBP-HexS = N-terminal fusion of maltose binding protein to HexS, comp. = unlabelled competitor *pigA* promoter DNA.

firmed a previous report that the HexS protein binds to *pigA* promoter DNA *in vitro* using a recombinant version of HexS in an EMSA assay [8], but using recombinant HexS protein that had been cloned from the CMS376 genome (**Figure 3(b)**).

The major goal of this study, to test whether HexS is required for inhibition of the secondary metabolite, prodigiosin, by glucose was achieved. Genetic analysis implicated the HexS transcription factor in the glucose inhibition of secondary metabolite phenotype. Interestingly, unlike the other glucose insensitive mutants isolated [5], *hexS* mutation neither prevented early medium acidification nor prevented glucose oxidation by glucose dehydrogenase.

Together results suggest that HexS transcriptional control of *pigA-N* is an important factor in inhibiting prodigiosin production in glucose-rich medium. Given that HexS negatively regulates multiple secondary metabolites [8], and the data from this study, we propose that HexS may be the major regulator involved of the inhibition of secondary metabolites by glucose. Future experimentation will be aimed at determining whether HexS activity responds directly to changes in pH. We propose that mutation of LrhA-like regulators may be useful for production of desired secondary metabolites.

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