Temperature Regulation of the Heme Transport System in *Escherichia coli*

by
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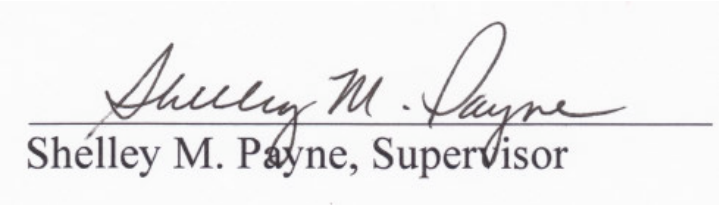
April 30th, 2010

A thesis submitted to the Faculty of the School of Biological Sciences at the University of Texas at Austin in partial fulfillment of the requirements for the degree of Bachelor of Science in Honors Biology



Abstract

Temperature regulation of the heme transport system is important, as it allows the microorganism to sense that it has entered the mammalian host environment of 37° C and adjust the expression of its iron-transport and virulence genes accordingly. It is known that expression of *shuA*, a gene encoding the outer membrane receptor that binds heme, is regulated by the amount of iron in the environment, yet little is known about the role of temperature in the regulation of this system. To study the effect of temperature on the activity of the *shuA* promoter, *E. coli* was transformed with a plasmid containing the *shuA* promoter fused to the reporter gene *lacZ* and transformed cultures were assayed for ß-galactosidase activity as an indirect measure of *shuA* promoter activity. Results suggest that *shuA* promoter activity is temperature regulated, with increased activity at 37° C compared to 30° C. The histone-like nucleoid structuring protein (H-NS) is a global regulator in *E. coli*, and it has been shown to respond to environmental signals, including osmolarity, temperature, and pH. To test the hypothesis that H-NS is mediating *shuA* promoter activity, gene expression was measured in an *hns* mutant and wild-type strain. Results indicate that H-NS is partially, but not solely, responsible for the temperature regulation of *shuA*. In a separate investigation, the possibility of exploiting the presence of heme transport systems in pathogenic bacteria to deliver toxic compounds to the pathogetn was tested. The toxicity of MGd and MGd-18-crown-6 texaphyrin compounds to *E. coli* expressing the heme transport system was examined. Results suggest that texaphyrin compounds are not toxic to *E. coli* growth.

Table of Contents

Introduction

Iron's importance in bacterial growth and virulence

Iron plays a critical role in the growth of nearly all bacterial pathogens (Neilands, 1981). This essential nutrient is involved in many cellular processes, including electron transport, nucleotide biosynthesis and the tricarboxylic acid cycle. Iron's reactivity is a result its ability to alternate between a ferric (Fe^{3+}) and a ferrous (Fe^{2+}) state, with a redox potential that ranges from +300 mV to –500 mV (Braun and Killmann, 1999).

Studies have shown that iron acquisition is strongly related to bacterial virulence in a variety of microbial pathogens. Bacterial strains with mutations in iron transport genes are often outcompeted by wild-type strains during infection, and many exhibit decreased virulence (Reeves et. al., 2000; Velayudhan et al., 2000; Rodriguez and Smith, 2006; Boulette and Payne, 2007; Fisher et. al., 2009).

The heme transport system

Although microbial pathogens require iron within a range of 0.4μM to 4μM in order to survive (Weinberg, 1978), free iron in the mammalian host is maintained around 10^{-18}M due to its low solubility at pH 7 (Braun and Killmann, 1999). The presence of host high affinity iron-binding proteins, including transferrin, lactoferrin, and hemoglobin, also limit iron's accessibility to invading pathogens.

In order to survive the iron-restricted host environment, bacterial pathogens have evolved several sophisticated iron transport and utilization systems. Common methods to acquire host

iron include the production of high affinity iron chelators (siderophores, hemophores), as well as the direct acquisition of iron through specific bacterial membrane transport systems.

One iron transport system common to both *Escherichia coli* and *Shigella dysenteriae* is the heme transport system (Figure 1). Heme is a metalloporphyrin ring containing a single iron atom. Heme plays a critical role in several biological processes, most notably the storage and transport of O_2. Heme also is involved in respiration, microRNA processing, cell differentiation, gas sensing, and the regulation of gene expression (Tong and Guo, 2009).

In *S. dysenteriae*, the heme transport system is encoded by the *shu* locus, which contains eight genes. ShuA is the outer membrane receptor that binds heme (Mills and Payne, 1997). ShuT is a periplasmic heme-binding protein (Eakanunkul et al., 2005), and ShuS is a cytoplasmic heme-binding protein that may function as both a heme storage protein and as a DNA-binding protein (Wilks, 2001). A recent study has revealed that ATP hydrolysis and heme transport are coupled from ShuT to ShuS (Burkhard and Wilks, 2008). Like ShuA, both ShuT and ShuS are required for efficient growth when heme is the sole iron source (Wyckoff et. al., 2005; Burkhard and Wilks, 2008). Based on their sequences, ShuT, along with ShuU and ShuV, is predicted to be a periplasmic ABC transporter system that translocates heme across the cytoplasmic membrane (Crosa et. al., 2004). Additional genes contained in the *shu* locus include *shuW*, *shuX*, and *shuY*, though their function in heme transport remains unclear.

Previous studies have reported that *Shigella* and *E. coli* belong to the same genetic species, and that *Shigella* strains should be considered clones of *E. coli*, with unique host specificity and pathogenesis (Ochman et. al., 1983; Pupo et. al., 1997; Lai et. al., 1998). Thus, it is not surprising that the heme-transport system in *E. coli* is nearly identical to that of *S. dysenteriae*. In fact, ShuA is 99.5% identical to ChuA, the heme receptor in *E. coli* O157:H7

(Torres and Payne, 1997). Because of the similarity between these heme transport systems, the *E. coli chuA* gene will be referred to as *shuA* in this thesis.

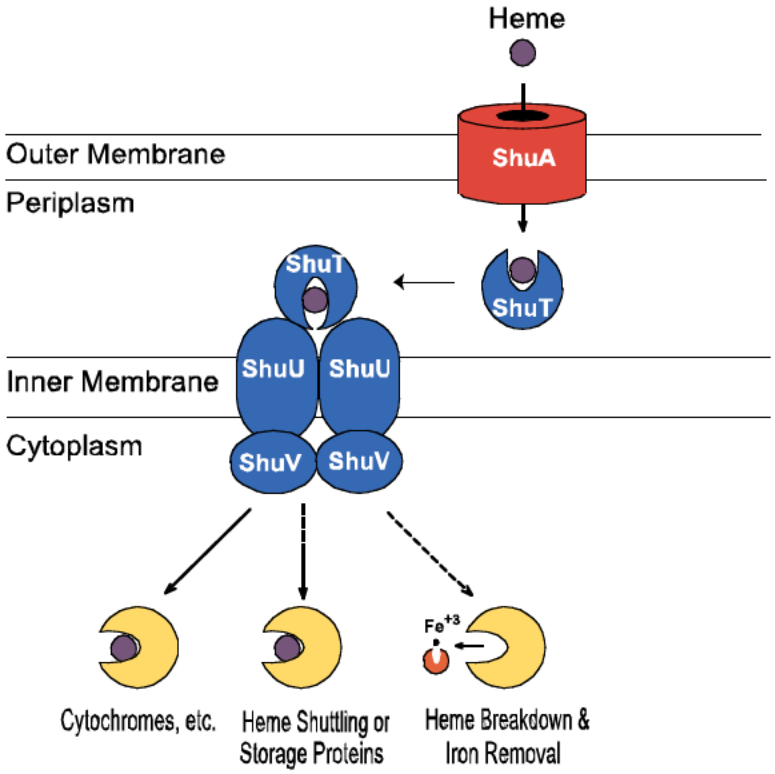


FIGURE 1: The heme-transport system of *S. dysenteriae* consists of ShuA, the outer membrane protein that binds heme. ShuT, ShuU, and ShuV function as an ABC transporter system that transports heme across the cytoplasmic membrane. This system also consists of ShuS, a cytoplasmic heme-binding protein, as well as proteins with unknown functions, including ShuW, ShuX, and ShuY (not shown). This figure was supplied by E. Wycoff.

Regulation of the heme transport system

Prior studies have shown that *shuA* is regulated by the concentration of iron, with high iron levels causing repression (Mills and Payne, 1995), and that this iron regulation is mediated by Fur (Mills and Payne, 1997). Fur (Ferric Uptake Regulator), whose DNA-binding activity is Fe^{2+}-dependent, is a transcriptional repressor of iron-regulated promoters. In the standard model of activity, Fur dimmers bind the divalent iron during high iron concentrations, causing the protein to alter its configuration. Fur then binds target DNA sequences (Fur boxes) within the promoter and represses expression of target genes (Escolar et. al., 1999).

Previous studies in our laboratory have suggested that expression of the heme transport system is also regulated by temperature. In *S. dysenteriae*, activity of the *shuA* promoter was higher at 37ºC compared to 30ºC (Dr. Sanchita Bhandra, unpublished data). This observation led to the hypotheses that 1) activity of the *shuA* promoter in *E. coli* is also regulated by temperature, and that 2) the differential expression of *shuA* at 37ºC compared to 30ºC is likely due to regulatory proteins interacting with the *shuA* promoter. The histone-like nucleoid structuring protein (H-NS) is a good candidate to regulate the heme transport system of *E. coli* because it exerts broad and pleiotropic effects on gene regulation in response to environmental stimuli, including temperature (Atlung and Ingmer, 1997).

Histone-like nucleiod structuring protein (H-NS)

Similar to a eukaryotic histone protein, H-NS is a DNA-binding protein that is able to condense DNA (Spassky et. al., 1984). H-NS has a unique C-terminal DNA-binding domain without any structural similarity to other DNA-binding proteins, as well as an N-terminal dimerization domain (Shindo et. al., 1995).

H-NS is known to repress transcription of a number of genes encoding virulence factors, ribosomal RNAs, and outer membrane porins (Porter and Dorman, 1994; Tippner et. al., 1994; Suzuki et. al., 1996). There also is evidence that H-NS may induce expression of target genes (Bertin et. al., 1994).

Interestingly, many of the target genes repressed by H-NS are regulated by environmental conditions, including osmolarity, pH, and temperature (Atlung and Ingmer, 1997). Previous studies provide evidence that genes encoding particular virulence factors and pili are regulated by both temperature and H-NS in gram-negative bacteria, though the role of H-NS in this thermoregulation remains unclear (Jordi et. al., 1992; Tobe et. al., 1993).

S. dysenteriae* and *E. coli

Shigella dysenteriae are gram-negative, rod-shaped bacteria that cause shigellosis, an acute intestinal infection, in the human host. Symptoms of shigellosis range from watery diarrhea to severe inflammatory bacillary dysentery. *S. dysenteriae* and other *Shigella* species are responsible for five to fifteen percent of diarrheal infections and over 1.1 million deaths worldwide (Kotloff et. al., 1999).

Shigella transmission occurs by the fecal-oral route, through ingestion of contaminated food or water. *Shigella* is highly infectious—only 10 to 100 microorganisms are necessary to cause disease (DuPont et. al., 1989). After surviving the acidic environment of the stomach with the use of acid resistance systems (Gorden and Small, 1993), *Shigella* travel to the large intestine where they establish infection. In order to cross the epithelial cell layer of the intestine, *Shigella* trigger their uptake by microfold cells (M cells) (Wassef et. al., 1989). M cells deliver bacteria to the intraepithelial pocket, where they are engulfed by resident macrophages. Within four hours

of infection, the bacteria induce apoptosis of the macrophage cells, causing their release into the sub-mucosa (Zychlinsky et. al., 1996). *Shigella* then invade the epithelial cell layer from the basolateral side and spread to adjacent epithelial cells.

Oral rehydration, along with antibiotics, is the standard treatment for shigellosis. At this time, there are no vaccines available to protect against infection. Current studies, however, aim to develop live vaccine strains that will stimulate mucosal immunity (Jennison and Verma, 2004).

In the United States around 73,000 cases of *E. coli* infection occur each year, with an estimated 61 deaths. Intestinal infection by one serotype, *E. coli* O157:H7, is characterized by severe abdominal pain and watery diarrhea. Though the illness is typically self-limited, serious complications, including hemolytic uremic syndrome (HUS), neurologic damage, and thrombotic thrombocytopenic purpura (TTP) are possible in patients that are immunocompromised or elderly. This pathogenic *E. coli* shares several characteristics with *Shigella* species, including the presence of the *Shu* heme transport system. Common transmission of *E. coli* occurs by ingestion of tainted food, particularly undercooked beef, or by drinking contaminated water or milk (Shetty et. al., 2009).

Texaphyrins

Texaphyrins (Figure 2) are metal-coordinating expanded poryphyrins, aptly named for their size and semblance to the star in the Texas state flag (Harriman, 1989). Because of their similarity in structure to heme, these molecules may act as either toxic agents or as barriers to the uptake and utilization of heme in bacteria with heme-transport systems. In addition, these compounds may be good candidates for novel antibiotics, as they have been shown to be non-

toxic in humans (Rosenthal, 1999). In this investigation, two texaphyrin compounds were tested for toxicity to *E. coli* containing the *shu* genes: MGd and MGd-18-crown-6 texaphrin. MGd-18-crown-6 is similar to MGd, except that it contains an 18-crown-6 moiety. This structural difference may have a positive effect on this compound's cellular uptake properties due to its ability to form cationic complexes with alkali ions and tertiary amines.

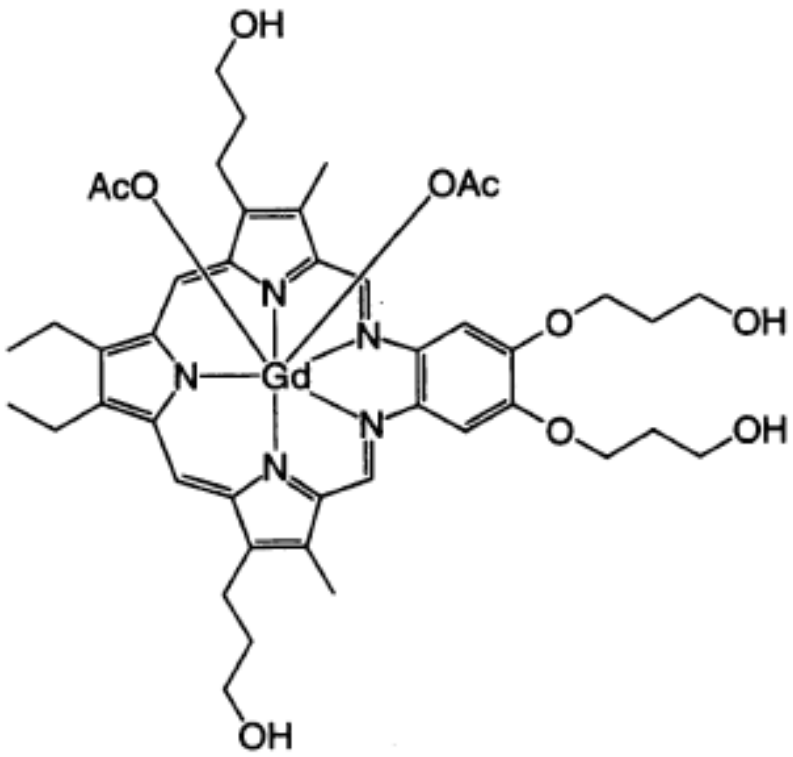


FIGURE 2: Gadolinium (III) Texaphyrin (MGd), a representative of the Texaphyrin class of compounds (Young, 1996.)

Materials and Methods

Bacterial strains and plasmids: Bacterial strains and plasmids used in this study are listed in Table 1.

TABLE 1:

Strain/Plasmid	Relevant Characteristics	Source
***E. coli* strains**:		
CA7027	*lac*$^-$	S.M. Payne
CA7027 Δhns	Keio mutation in *hns*	A.L. Gore
1017	HB101*ent*::Tn5	S.M. Payne
Plasmids:		
pQF50	Vector	
pSB15	*shuA* upstream region cloned into PQF50	S. Bhadra
pSHU12	pSHU12 allows for heme utilization in *E. coli*	S.M. Payne

Media: Strains were grown aerobically on L agar and cultured in L broth at either 37°C or 30°C. L broth contains 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl per liter. L broth pH was adjusted to 7.0 using NaOH. To prepare L agar, 15 g of Bacto-agar was added to each liter of L broth.

Preparation of Frozen Competent *E. coli* cells: The *E. coli* strain to be made competent for transformation was grown overnight in L broth. On the next day, the culture was chilled on ice for 15 minutes. Then, the culture was centrifuged at 8,000 x g for 10 minutes at 4°C. The cell pellet was resuspended in cold 100 mM $CaCl_2$ at one-half the original volume, and centrifuged as before. This process of was repeated until the culture was at one-tenth of its original volume, at which point the cell pellet was resuspended in cold 100 mM $CaCl_2$ solution containing 10% glycerol and stored in 120 µl aliquots at -80°C.

Transformation of *E. coli* cells: For transformation, 1 µl of DNA was added to the 120 µl of competent cells, which were thawed prior to DNA addition. The transformation reactions were then heat-shocked for 42°C for 1.5 minutes and further incubated on ice for 10 minutes. After this time, the transformation reactions were diluted with 900 µl of L broth and incubated for 1 hour at 37°C. An aliquot of 100 µL of cells, as well as 100 µl aliquot of tenfold concentrated cells, were spread onto selective agar medium and incubated overnight.

Measurement of ß-galactosidase activity: Expression of the *lacZ* reporter gene was determined using the Miller method (Miller, 1972). Following overnight incubation of *E. coli* at either 37°C or 30°C, bacteria in 1 ml of culture were pelleted by centrifugation for 5 min at 3,000 x g. The pellet was resuspended in 1 ml of Z buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$-$7H_2O$, 38 mM ß-mercaptoethanol). Following the adjustment of OD_{600} to 0.2-0.7, 400 µl of cell suspension was diluted with 400 µl of Z buffer. Cells were then permeabilized with 50 µl of 0.1% SDS and 100 µL of chloroform, and incubated at 30°C for 15 min. Then, 160 µl the substrate o-nitrophenyl- ß-D-galactopyranoside (ONPG) was added at a

concentration of 4 mg/ml to the reaction, which was incubated at 30°C until yellow color developed. The addition of 400 µl of 1 M Na_2CO_3 was used to stop the reaction. Following centrifugation for 2 min, the OD_{420} and OD_{550} were recorded. Activity of ß-galactosidase was calculated using the following formula:

$[1000(OD_{420} - 1.75(OD_{550}))]/(t)(V)(OD_{600})$

t = time of reaction in minutes, V = volume of cell suspension used in reaction

Texaphyrin Screening:

E. coli (strains 1017 and 1017 pSHU12) was incubated overnight. 100 µl of overnight culture was diluted in 5 ml of L-broth, and 100 µl of this diluted culture was added to 2.5 ml of soft agar. This soft agar overlay was poured onto L-agar plates and allowed to harden at room temperature. 5 µl of MGd texaphyrin solution (1 mg/ml) was spotted onto to the plates, as well 5 µl of MGd-18-crown-6 solution (1 mg/ml). To measure the sensitivity of *E. coli* strains to MGd and MGd-18-crown-6 texaphyrins, the zone of inhibition (ZOI) surrounding each texaphyrin compound was measured following overnight incubation.

Results

shuA expression is temperature mediated in *E. coli*

To assess the effect of temperature on *shuA* promoter activity, *E. coli* was transformed with either pSB15, a plasmid containing the entire *shuA* promoter sequence fused to the reporter gene *lacZ*, or pQF50, a control vector. After overnight incubation at either 37°C or 30°C, the transformed cultures were assayed for ß-galactosidase activity as an indirect measure of *shuA* promoter activity.

Compared to cultures incubated at 30°C, cultures incubated at 37°C exhibited an increase in ß-galactosidase activity (68 units vs. 43.5 units) when transformed with pSB15 (Figure 3). This result supports the hypothesis that activity of the *shuA* promoter is temperature responsive, with higher levels of activity at 37°C compared to 30°C.

H-NS partially mediates temperature mediation in *E. coli*

To assess the contribution of H-NS to the temperature-dependent regulation of *shuA* promoter activity, ß-galactosidase reporter assays were repeated in an *E. coli hns* mutant and wild-type *E. coli* strain. After overnight incubation at either 37°C or 30°C, the cultures were assayed for ß-galactosidase activity as an indirect measure of *shuA* promoter activity.

Whereas the wild-type *E. coli* strain had a four-fold increase in ß-galactosidase activity from 30° C to 37° C (15.8 units vs. 56.7 units), the *hns* mutant only exhibited a two-fold increase in activity (12.3 units vs. 25.9 units) (Figure 4). This result is consistent with the hypothesis that H-NS may be mediating expression of *shuA* with respect to temperature.

Texaphyrin compounds do not inhibit growth of *E. coli*

To test the hypothesis that MGd and MGd-18-crown-6 texaphyrins are toxic to *E. coli*, texaphyrin compounds were spotted onto soft agar overlay plates containing *E. coli*, and the resulting zones of inhibition were measured. For both MGd and MGd-18-crown-6 texaphyrin compounds, zones of inhibition were not observed for either *E. coli* strain tested.

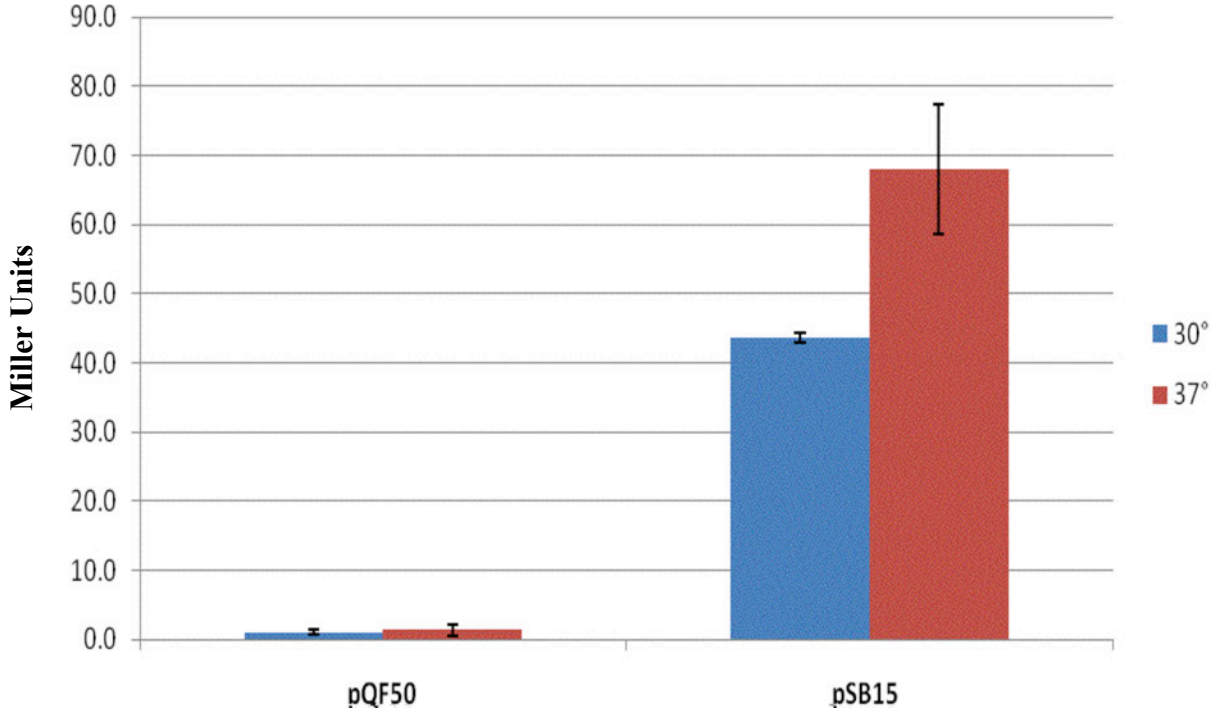


FIGURE 3: *E. coli* strain CA7027 was transformed with either pQF50 or pSB15 and incubated overnight at either 37°C or 30°C. Activity of *shuA* promoter was measured using the ß-galactosidase assay according to the Miller method. When transformed with plasmid pSB15, overall activity was increased and temperature regulation of expression was exhibited. Crossbars represent +/- one standard deviation from the mean of three trials.

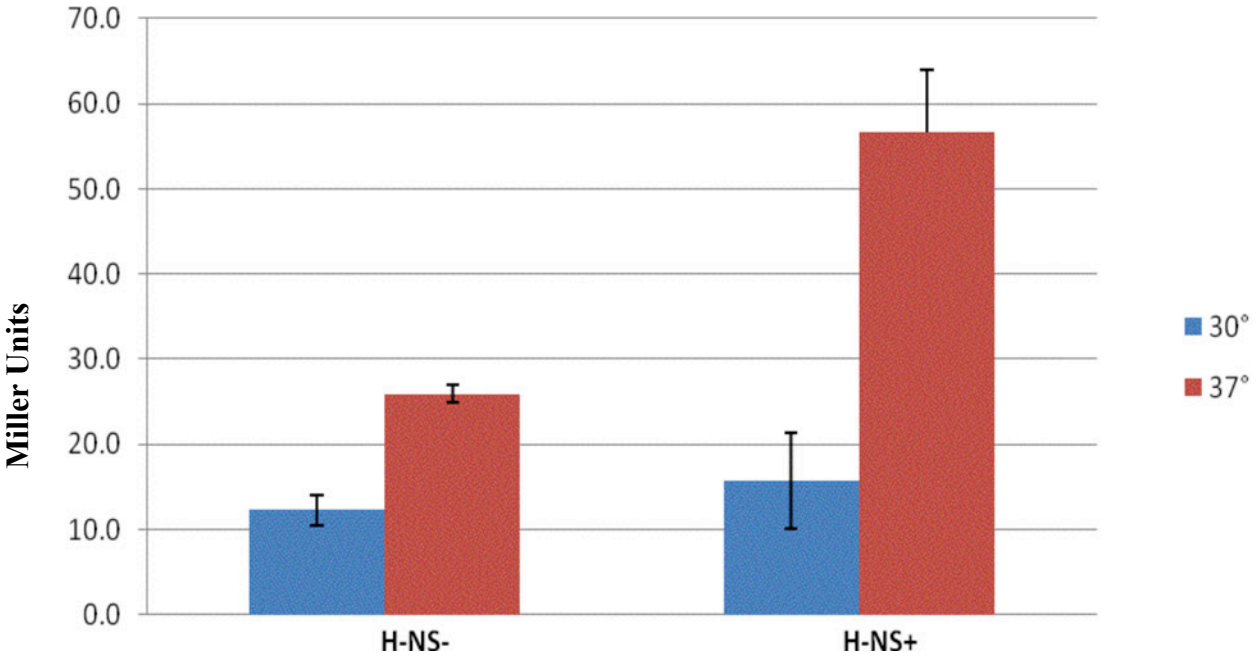


FIGURE 4: An *hns* mutant *E. coli* strain and a wild-type *E. coli* strain were transformed with pSB15 and incubated overnight at either 37°C or 30°C. Activity of *shuA* promoter was measured using the ß-galactosidase assay according to the Miller method. Although the *hns* mutant exhibited partial repression at 37°C compared to the wild-type strain, temperature regulation still occurred. Crossbars represent +/- one standard deviation from the mean of three trials.

Discussion

An invading pathogen must be able to acquire iron in order to successfully establish infection and cause disease in the host. Thus, there is strong selective pressure for bacterial pathogens to evolve iron-transport mechanisms to acquire this essential nutrient from the host. Many pathogens, including *Shigella dysenteriae* and *Escherichia coli*, have evolved heme transport systems, as heme is the most abundant source of iron in the mammalian host. ShuA is the outer membrane receptor for the *S. dysenteriae* heme transport system, and previous studies have shown that expression of *shuA* is regulated by the concentration of iron in the environment, with high iron levels causing repression (Mills and Payne, 1995). Prior studies in our laboratory also suggest that expression of *shuA* is regulated by temperature in *S. dysenteriae* (Dr. Shanchita Bhadra, unpublished data).

The results of this study show that the promoter activity of *shuA* is regulated by temperature in *E. coli*, and that H-NS is partially, but not solely, responsible for mediating *shuA*'s differential expression at 37°C compared to 30°C. Regulation of the heme transport system by temperature would allow *E. coli* to sense that it has entered the human host environment of 37° C and, as a result, *E. coli* can adjust the expression of its iron-transport and virulence genes. Furthermore, the use of temperature to regulate iron-transport genes involved in the acquisition of mammalian-specific sources of iron, such as heme, is a good strategy for two reasons, 1) temperature of the host is very different than that of the external environment, and 2) temperature, specifically 37°C, is a consistent environmental signal among human hosts.

H-NS mediates the temperature regulation of virulence gene expression in both *Shigella* and *E. coli* (Jordi et al., 1992; Tobe et al., 1993), so it is not unreasonable to suspect that H-NS may

play a role in the temperature regulation of the heme-transport system as well. In previous studies of temperature regulation, *hns* mutants exhibited derepression of target-gene expression at low temperatures. This result, however, was not observed in the thermoregulation of *shuA* expression in an *E. coli hns* mutant at a low temperature; the promoter activity of *shuA* was nearly the same in both wild-type and *hns* mutant strains at 30°C. A difference in *shuA* promoter activity between the wild-type and *hns* mutant was only observed at 37°C, with higher activity in the wild-type strain than in the *hns* mutant. These data suggest that H-NS may be regulating *shuA* expression by a mechanism other than repression at low temperatures.

Several hypotheses have been proposed to address the mechanisms by which H-NS affects expression of target genes. One model suggests H-NS prevents RNA polymerase from interacting with target gene promoters where H-NS binding sites overlap with promoter elements (Ueguchi and Mizuno, 1993). A second model suggests that H-NS affects the accessibility of gene promoters to RNA polymerase through alterations in DNA supercoiling (Hulton et. al. 1990), though more recent studies provide evidence that there is not a strong correlation between H-NS mediated DNA supercoiling and target gene expression (Kawula and Orndorff, 1991; Kano et. al., 1993). A third model proposes that H-NS acts locally to constrain negative supercoils, thereby repressing gene expression (Goransson et. al., 1990; Tupper et. al., 1994). In addition, H-NS may act indirectly through a second mediator, such as RNA polymerase sigma factor (s^s), to affect gene transcription (Hengge-Aronis, 1996).

The mechanism by which H-NS mediates the thermoregulation of *shuA* remains uncertain. It is clear from this investigation's results, however, that H-NS is not the only regulator involved in *shuA* promoter activity, as some thermoregulation of *shuA* remains evident in an *hns E. coli* mutant.

Investigating gene regulatory systems is of great importance, as these studies will allow for the development of new antimicrobial compounds that target specific components of these systems. Discovering alternatives to traditional antibiotics is especially critical as drug-resistant strains become more commonplace.

In a separate study, the toxicity of MGd and MGd-18-crown-6 texaphyrin compounds to *E. coli* expression the heme transport system was examined. Zones of inhibition were not observed for either MGd or MGd-18-crown-6 texaphyrin, suggesting that these compounds are not toxic to *E. coli*. One explanation for this lack of toxicity may be that texaphyrins are unable to be transported into *E. coli* cells by the heme-transport system due to size and structural differences between these compounds and heme. Texaphyrin compounds, however, may still affect binding and utilization of heme via competitive binding to ShuA. Although this hypothesis was not tested, it would be an interesting topic for future investigations.

Future directions

Future research will be focused on identifying other proteins that may act in conjunction with H-NS to regulate the heme transport system in *E. coli*. The technique of transposon mutagenesis will be used as a screening tool for additional regulatory proteins. A library of transposon mutants in *E. coli hns* mutant *lacZ* fusion strains will be created and *shuA* promoter activity will be detected in these strains by screening for blue colonies on an agar medium containing the indicator x-gal. By using blue-white x-gal screening based on expression of *lacZ*, mutants exhibiting repressed expression at 37°C or induced expression at 30°C will be identified. These mutated genes will then be sequenced to identify additional genes that may contribute to the temperature regulation of *shuA* in *E. coli*. After successful identification of these proteins, the interaction between the identified regulatory proteins, H-NS, and the *shuA* promoter will be investigated using electrophoretic mobility shift assays and DNase footprinting to determine the binding site of the regulatory proteins within the *shuA* promoter. Additional affects of these proteins on *shuA* expression will be measured using quantitative RT-PCR. Promoter elements critical for interaction with temperature-responsive regulators will be defined using site-directed mutagenesis and selective deletions within the *shuA* promoter. With the results from these experiments, both the transcription proteins responsible for temperature regulation and the site of their interaction within the *shuA* promoter will be characterized. These findings, in turn, will contribute to the overall understanding of the regulation of the heme transport system in *E. coli*.

Acknowledgements

I would like to thank my thesis advisor Dr. Shelley M. Payne for introducing me to the wonderful world of genetics, and for providing me with support throughout my undergraduate education. I would also like to thank Dr. Erin R. Murphy for her patience and guidance during my first semester in the laboratory. Thank you to all of the members of the Payne lab for helping me with my project, from aiding me in my search for lost strains in the lab freezer to teaching me how to set-up PCR reactions.

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