Published in final edited form as:

Microb Pathog. 2016 March ; 92: 1–10. doi:10.1016/j.micpath.2015.12.010.

Characterization of the general stress response in *Bartonella henselae*

Nhan Tu, Amorice Lima, Zahra Bandiali, and Burt Anderson*

Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA

Abstract

Bacteria utilize a general stress response system to combat stresses from their surrounding environments. In alpha-proteobacteria, the general stress response uses an alternate sigma factor as the main regulator and incorporates it with a two-component system into a unique regulatory circuit. This system has been described in several alpha-proteobacterial species, including the pathogens *Bartonella quintana* and *Brucella abortus*. Most of the studies have focused on characterizing the PhyR anti-anti-sigma factor, the NepR anti-sigma factor, and the alternate sigma factor. However, not enough attention is directed toward studying the role of histidine kinases in the general stress response. Our study identifies the general stress response system in *Bartonella henselae*, where the gene synteny is conserved and both the PhyR and alternate sigma factor have similar sequence and domain structures with other alpha-proteobacteria. Our data showed that the general stress response genes are up-regulated under conditions that mimic the cat flea vector. Furthermore, we showed that both RpoE and PhyR positively regulate this system and that RpoE also affects transcription of genes encoding heme-binding proteins and the gene encoding the BadA adhesin. Finally, we identified a histidine kinase, annotated as BH13820 that can potentially phosphorylate PhyR.

Keywords

Bartonella henselae; general stress response; two-component system; alternate sigma factor; gene regulation; histidine kinase

1. Introduction

Bacteria are under constant stress from their surrounding environment, which can include a variety of stressors such as temperature, pH, osmotic shock, nutrient availability, and many other diverse environmental stresses that can threaten their survival [1]. For pathogenic bacteria in which their surrounding environment is different host conditions, the stresses that

*Corresponding author: Burt Anderson, PhD, Department of Molecular Medicine, MDC7, Morsani College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., Tampa, FL 33612, Telephone: (813) 974-2608, banderso@health.usf.edu.

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.

they encounter can come from the host's immune system. At times the bacteria can be in direct competition with the natural flora inside the host, as well as the body temperature of the host, the pH inside the host cell, stress from reactive oxygen species imposed upon the bacteria by the host cell, in addition to the limited available nutrients that can support the bacteria's survival [2]. Due to the assault of multiple stress stimuli, the bacteria must be capable of combating these stresses and eventually adapt to the surrounding environment in order to survive or even to infect the host cells. Thus, a general stress response system (GSR) has been acquired by various bacterial species that can protect themselves against the stresses that threaten their survival. Depending on the bacterial species, the GSR systems can be diverse in terms of their regulatory mechanisms. Two very well characterized examples of the GSR system have been identified in *Escherichia coli* and *Bacillus subtilis*. In both systems, the main regulator of the stress response system is the alternate sigma factors RpoS and SigB, respectively. The regulatory mechanisms of these systems have been well studied, with both systems reliant on the regulation of the alternate sigma factor either at the transcriptional or translational level, via proteolysis, or by their cognate anti-sigma factor [3, 4].

The general stress response in alpha-proteobacteria has been shown to involve a partner switching mechanism whereby an anti-sigma factor (termed NepR) either binds to an extracytoplasmic function (ECF) sigma factor (termed σ^{EcfG}) and inhibits σ^{EcfG} activity or it binds to an anti-anti-sigma factor (termed PhyR), allowing σ^{EcfG} to interact with the core RNA polymerase (see [5, 6] for review). The unique property of this GSR system that is not found in other bacteria outside of the alpha-proteobacteria is the incorporation of the ECF sigma factor with another gene regulatory mechanism, the two-component system (TCS). The components of a TCS include a membrane-bound histidine kinase (HK) and its cognate response regulator (RR). Both proteins communicate via a phosphoryl transfer from the HK to the RR. The HK acts as a sensor protein and auto-phosphorylates a conserved histidine residue on its kinase domain upon stimulus activation. Following activation, the phosphoryl group is transferred from the HK to a conserved aspartate on the N-terminal receiver domain of the RR. The effector function of RRs is generally to bind to DNA and affect gene transcription [7]. In the case of the TCS in the GSR system of alpha-proteobacteria, the anti-anti-sigma factor PhyR is a response-regulator like protein that gets phosphorylated by a histidine kinase. However, the effector domain of PhyR cannot bind to DNA since it lacks the necessary residues for DNA-binding [8]. Instead PhyR regulates the activity of the NepR anti-sigma factor via protein-protein interaction [5]. The diverse environmental cues that determine if the GSR is activated include temperature, desiccation, oxidative, osmotic stress, acid stress, UV, ethanol, and hemin [8–14]. This stimulus-mediated activation through a sensor kinase results in phosphorylation of PhyR so that it can then bind NepR and thus preventing NepR from binding and inactivating σ^{EcfG} . In general, the specific environmental cues responsible for activating the GSR in alpha-proteobacteria vary with the specific bacterium. Furthermore, the specific sensor HK that responds to these cues has not been completely characterized. Putative HK genes are found adjacent to the genes in the GSR system in almost all alpha-proteobacteria and it has been shown that some of these HK genes are involved in the phosphorylation and/or dephosphorylation of PhyR [12, 14–16]. Recently, this novel GSR system was described in *B. quintana* and shown to be involved in

the adaptation of this bacterium to the lower temperature and higher heme concentrations of the arthropod vector for this bacterium, the human body louse. However, the GSR system has not been characterized in other *Bartonella* species and the roles of the two histidine kinases in the general stress response in *Bartonella* remain unexplored [9].

The genus *Bartonella* consists of Gram-negative, arthropod vector-borne facultative intracellular bacteria that infect a wide range of hosts [17, 18]. While humans are the reservoir hosts for *B. bacilliformis* and *B. quintana*, cats are the natural host for *Bartonella henselae* (*Bh*) in which they usually cause an asymptomatic intra-erythrocytic infection. The organism, however, can be transmitted between cats by the cat flea (*Ctenocephalides felis*), and from infected cats to humans by scratching. Immunocompetent individuals infected with *Bh* typically suffer from cat scratch disease (CSD), which is a self-limiting infection. In contrast, immunosuppressed individuals infected with *Bh* can develop systemic infections including bacillary angiomatosis (BA) and bacillary peliosis (BP), characterized by vasoproliferative tumor-like lesions on the skin and liver, respectively [17, 19]. These lesions are a result of proliferation of vascular endothelial cells found in close association with aggregates of *Bh* [20]. As a zoonotic bacterium, *Bh* must be able to adapt to very diverse environments such as the cat flea vector where the temperature is low and after a blood meal the heme is at toxic levels. In contrast in the vertebrate host the temperature is higher and heme availability is restricted [9]. In other bacteria this rapid adaptation has been shown to be the results of a global or general stress response network. The general stress response described in *B. quintana*, the agent of trench fever, was found to be responsible for the bacteria's survival and adaptation in the body louse, which is rich with toxic heme [9]. In addition, it was shown that the general stress response sigma factor RpoE is responsible for infection of mice in *Brucella abortus* and thus, is necessary for the bacteria to survive the stresses in mammalian cells [11]. We have shown that the response of *Bh* to a high temperature shock involves the high temperature requirement (HtrA) stress response protein. The gene encoding HtrA is transcribed from two independent promoters, one of which is thought to be recognized by an alternate ECF sigma factor, RpoE [21]. Such ECF sigma factors recognize distinct promoter sequences altering the specificity of RNA polymerase and thereby rapidly mediating shifts in gene expression patterns in response to environmental cues [22]. The role of alternate sigma factors in controlling gene expression and response to stress in *Bh* remains uncharacterized. Here, we present a description of the GSR system in *Bh* and the associated kinases responsible for phosphorylating PhyR.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Bh strain Houston-1 [23] was used as the wild-type parental strain upon which subsequent gene manipulations were performed. Bacteria were either cultured on heart infusion agar (Remel, Lenexa, KS) supplemented with 1% bovine hemoglobin (Remel, Lenexa, KS) (also known as chocolate agar) or in Schneider's insect medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Pittsburgh, PA) and incubated for 3–4 days at 37°C in the presence of 5% CO₂ [24]. The pNS2-derived expression vector, which has been shown previously to replicate in *Bh* [25], was used as a

backbone for construction of promoter reporter plasmids and introduced into *Bh* via electroporation [21]. For bacterial strains carrying the pNS2 plasmids, 50 µg/ml of kanamycin was supplemented to select for bacteria colonies that harbor the plasmid. All manipulations of *Bh* have been approved by the USF Institutional Biosafety Committee.

2.2 Construction of gene deletion mutants of the GSR genes

In-frame deletion mutants of the full length *rpoE* (BH13830), *phyR* (BH13850) and putative HKs (BH13820 and BH13860) were constructed in *Bh* Houston-1 using the two-step mutagenesis strategy described by Mackichan et al. [26]. Briefly, *Bh* genomic DNA was used as template for PCR to generate two fragments of the gene to be deleted using gene-specific primer pairs (Table S1). The first fragment contained an upstream region and included a small segment of the 5' part of the gene to be deleted whereas the second fragment contained a downstream region and a 3' segment of the gene. The two purified PCR products were used as templates for megaprimer PCR using only the forward primer from fragment 1 and the reverse primer from fragment 2. The resulting product was purified and ligated into the “suicide” plasmid pJM05 at the *Bam*HI restriction site [26].

The plasmids containing the deleted gene were transformed into DH12S *E. coli* and then transferred into *Bh* Houston-1 by trans-conjugation using a two-step allelic exchange strategy [26]. The pJM05 derivative integrated into the *Bh* chromosome by homologous recombination with the sequences flanking the target gene. Trans-conjugates were selected by plating on 5% rabbit blood agar supplemented with kanamycin (30 µg/ml), nalidixic acid (20 µg/ml), and cefalozin (2 µg/ml). The colonies were then counter-selected on agar containing 10% sucrose to promote excision of the integrated plasmid by a second crossover event resulting in replacement of the full-length gene with the truncated version. PCR was performed on genomic DNA isolated from kanamycin-sensitive sucrose-resistant colonies to confirm deletion of the specific gene. All mutants were verified by sequencing across the deleted region and by performing RT-PCR to ensure the absence of the mRNA from the mutant.

2.3 Bioinformatics analysis of the GSR genes

The domain structures of PhyR and the *Bh* ECF sigma factor RpoE were analyzed using the NCBI Conserved Domain Database [27–30]. The amino acid sequences of PhyR and RpoE were aligned with other alpha-proteobacterial species using the CLC Sequence Viewer program (CLC bio, Boston, MA).

2.4 Hemin exposure

Bh Houston-1 strain was cultured in Schneider's medium with or without addition of hemin to mimic the conditions of the cat flea vector (28°C with 5 mM hemin) or the vertebrate host (37°C with 0.05 mM hemin) [31]. The bacteria were also grown in Schneider's medium at the two temperatures without the addition of hemin. qRT-PCR analysis was performed to determine the transcription of the GSR genes under different conditions. The regulatory role of the GSR system in responding to toxic heme was analyzed through transcription of genes encoding heme-binding proteins (Hbps). Additionally, we wanted to determine whether the GSR system affects transcription of the *badA* gene, which encodes an adhesin responsible

for interacting with the host endothelial cells, in bacteria under hemin stress. As previously described, bacteria were cultured with 1 mM hemin at either 28°C or 37°C in the presence of 5% CO₂ [9]. qRT-PCR analysis was performed to determine the transcription of *badA* and *hbps* when the bacteria are assaulted with a toxic concentration of hemin.

To prepare hemin solution, hemin (Frontier Scientific, Logan, UT) was dissolved in 0.1N NaOH and filtered through a 0.22-μm filter to remove any undissolved hemin. The concentration of the hemin solution was then measured by reading the OD₅₇₂ of the solution. The hemin concentration was calculated based on the OD₅₇₂ of 5.5 of a 1 mM hemin solution [32]. The hemin solution was wrapped with aluminum foil to keep out light and stored at 4°C and used within a week.

2.5 RNA isolation

Bacteria were resuspended in Trizol reagent (Thermo Fisher Scientific, Grand Island, NY) and total RNA was extracted by phenol-chloroform extraction according to the manufacturer's instructions. The RNA was precipitated and the RNA pellet was resuspended in nuclease-free water. The RNA was subsequently treated with DNase using the Turbo DNA-free kit (Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's protocol to remove any contaminating DNA. The yield and quality of the extracted total RNA was evaluated by measuring the ultraviolet (UV) absorbance using the ND-1000 nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and by agarose gel electrophoresis.

2.6 cDNA synthesis and qRT-PCR

The DNase-treated RNA (1μg) was reverse transcribed into cDNA in a 20-μl reaction using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). Each reaction was performed in a 25 μl volume containing 2 μl of cDNA, 12.5 μl of 2X Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific, Grand Island, NY) and 300 nmol of each gene-specific primer (Table S1). All reactions were performed in triplicate, and 50S ribosomal protein L4 (*rplD*) was used as endogenous gene for normalization. Cycling parameters were 95°C for 3 min, followed by 40 cycles of 95°C for 10s and 60°C for 30s, 95°C for 45s, 55°C for 1 min. Melting curve analysis was performed to confirm that no primer dimers were amplified. Results were analyzed using the comparative C_T method (2^{-C_t}) [33].

2.7 Construction of promoter reporter plasmids

The *lacZ* reporter gene encoding the beta-galactosidase enzyme was previously ligated into the pNS2 plasmid downstream of the strong *trc* promoter [25]. The divergent *nepR* and *phyR* promoters were PCR amplified from *Bh* genomic DNA using specific primers (Table S1) and ligated into the pNS2 plasmid to replace the *trc* promoter at the *SalI* and *BamHI* restriction sites. The plasmid was introduced into DH12S *E. coli* and colonies were screened by kanamycin selection (50 μg/ml) and PCR screening. The plasmid was extracted from positive colonies and electroporated into *Bh* Houston-1. Colonies harboring the plasmid

were selected on kanamycin agar plates (25 µg/ml) and PCR screened to confirm the presence of the promoter and reporter gene in the correct orientation in the *Bh* plasmid.

2.8 Beta-galactosidase assay

Beta-galactosidase (β-gal) assays were performed using the protocol previously described by Miller [34]. Bacteria were grown in Schneider's liquid medium and collected by centrifugation. The pellets were resuspended in 1 ml of Z-buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄, and 0.05 M β-mercaptoethanol). 100 µl of the bacterial suspension was transferred to a 96-well microtiter plate and the OD₆₀₀ was measured using a plate reader. The remaining bacterial suspension was used for cell lysis with 50 µl chloroform and 25 µl of 0.1% SDS. The cells were lysed for 5 minutes at 28°C. Following lysis, 100 µl of the lysate was transferred to a 96-well microtiter plate. The substrate, o-nitrophenyl-β-D-galactoside (ONPG) (Sigma, St. Louis, MO), was used for hydrolysis by β-gal into galactose and ortho-nitrophenol, which exhibits a yellow color that can be measured colorimetrically at OD₄₂₀. 20 µl of ONPG was added to each well and the reaction was proceeded for 3 minutes before the reaction was stopped with 1M Na₂CO₃. The reaction was quantified at OD₄₂₀ to measure the absorbance of the yellow color and at OD₅₅₀ for cell debris scattering. The Miller Units were calculated using the following

formula: $1000 \times \frac{OD_{420} - (1.75 \times OD_{550})}{t \times v \times OD_{600}}$, where t = reaction time in minutes and v = volume of culture in ml.

2.8 *Bh* infection of human endothelial cells

In order to compare GSR deletion mutants to wild-type *Bh* for the ability to attach and invade endothelial cells, a gentamicin protection assay using the immortalized human microvascular endothelial cell line HMEC-1 was performed as previously described [35]. Briefly, endothelial cells were collected in MCDB 131 medium supplemented with 200 mM L-glutamine, 10% FBS, 20 µg/ml EGF, 500 µg/ml hydrocortisone, and 5 mM hepes and 10⁵ cells were allowed to attach overnight to each well of a 24-well plate. For infection, 10⁷ 3-day-old bacteria were collected, resuspended in supplemented MCDB 131 medium and added to each well containing attached endothelial cells to achieve a multiplicity of infection (MOI) of 100 and the infection was allowed to proceed for two hours at 37°C, 5% CO₂. Three replicates were performed for each bacterial strain. Following the infection period, the wells were washed three times with 1X PBS to remove bacteria that did not attach to or invade the cells. Subsequently, gentamicin was added to each well at a concentration of 200 µg/ml and incubated for one hour at 37°C, 5% CO₂ to target extracellular bacteria. The wells were washed three times with 1X PBS to remove remaining traces of gentamicin. The wells were treated with 0.1% saponin for 10 minutes to lyse the infected endothelial cells and liberate intracellular bacteria. Lysates were plated on chocolate agar and the plates were incubated at 37°C, 5% CO₂ for 7 days. Colonies that appeared on the plates were counted and the number of intracellular bacteria/well (1ml) were calculated.

3. Results and Discussion

3.1 Gene synteny within the GSR of *Bh* and other alpha-proteobacteria

In this study, we show that the GSR system exists in *Bh* and exhibits the conserved gene synteny described of the GSR genes previously in which *nepR* and *phyR* are divergently arranged and *rpoE*, the gene encoding σ^{EcfG} , is located downstream of *nepR* (Fig. 1) [5, 6]. Two genes in the vicinity that encode HKs, one harboring a HisKA_2 domain and the other a HWE_HK domain, are downstream of *rpoE* and *phyR*, respectively. It has been shown that some of these HKs either phosphorylate or de-phosphorylate PhyR [15, 16, 36].

3.2 RT-PCR analysis of GSR transcripts

We wanted to determine if this conserved arrangement of GSR genes resulted in the two divergent transcripts that have been described in other alpha-proteobacteria were also present in *Bh* by performing RT-PCR of the GSR gene locus. Using primers to amplify the products that would include the end of one gene, the intergenic region, and the beginning of the next gene, we wanted to ensure that the genes are transcribed on the same transcript. We also designed primers that would amplify the 5' end of *nepR*, the intergenic region, and the beginning of *phyR* to determine if there is an RNA species that spans the region between the expected two separate divergent transcripts (Fig. 2). Following RT-PCR, we separated the PCR product using gel electrophoresis. For each primer pair an amplicon of the expected size could be seen in the lane containing the RNA with reverse transcriptase (lane 1) that has the same size as the genomic DNA control (lane 4), while no product was seen in the RNA without reverse transcriptase (lane 2) or the no template control (lane 3), indicating that the product seen in lane 1 is not due to DNA contamination. The RT-PCR results suggest that the HisKA_2 gene, *rpoE*, and *nepR* are located on a single transcript, while *phyR* and the HWE_HK histidine kinase gene are localized together on a separate divergent transcript. These results are identical to what was reported for the GSR gene transcription in *B. quintana*, with *nepR*, *rpoE*, and one HK encoded on one transcript and *phyR* and the remaining HK encoded on the other transcript [9]. Surprisingly, we observed an amplification product when we used primers that would target the intergenic region between *nepR* and *phyR* (**Primer pair P4a and P4b**, Fig. 2, **P4, lane 1**). The results were not what we expected since we hypothesized that *nepR* and *phyR* would be transcribed only on two separate divergent transcripts in *Bh* as reported for the closely related species *B. quintana* [9]. Based on the unexpected results of the RT-PCR, it appears that both *nepR* and *phyR* are transcribed together on a third separate mRNA that spans the region between these two divergent genes and this transcript may also include one or more of the other GSR genes.

3.3 PhyR and σ^{EcfG} amino acid sequences and domain structures are conserved in *Bh*

We wanted to compare the amino acid sequence and domain structure of *Bh* PhyR and RpoE to that of other alpha-proteobacterial species (Fig. 3). When we aligned the *Bh* PhyR amino acid sequence with PhyR in other alpha-proteobacteria, we found that PhyR has the highest identity to PhyR in *B. quintana* (96.77% identity), which is expected due to the two species being closely related as members of the *Bartonella* genus. Compared to the other alpha-proteobacteria, *Bh* PhyR sequence is less conserved (the lowest is 49.10% identity to *Bradyrhizobium diazoefficiens*) but this is probably due to the more distant lineage of *Bh* to

the other alpha-proteobacteria. Overall, there is still a high degree of conservation among the alpha-proteobacterial PhyR based on the sequence alignment and the percent identity calculation. Like other alpha-proteobacteria, *Bh* PhyR contains a C-terminal receiver (REC) domain, which is the domain where phosphoryl transfer occurs (Fig. 3a). This organization differs from typical RRs where the REC domain is N-terminal [7]. Another observed difference of PhyR and other RRs is the N-terminal sigma factor-like domain of PhyR. This domain has sequence similarity to sigma factor proteins but rather is incapable of binding DNA due to the lack of DNA-binding residues [5]. Sigma factor mimicry is the proposed mechanism by which the ECF sigma factor is activated in the GSR system of alpha-proteobacteria. The similarity of the N-terminus of PhyR to the sigma factor can explain the binding competition of both PhyR and RpoE for NepR.

The RpoE protein of *Bh*, on the other hand, belongs to the σ^{ECF} family of sigma factors. σ^{ECF} are categorized into the sigma-70 group. Typical sigma-70 sigma factors, such as housekeeping sigma factors, contain four distinct regions (termed regions 1–4). Regions 2 and 4 bind to RNA polymerase and also assist the protein in recognizing the -35 and -10 promoter motif upstream of genes, respectively. Although a member of the sigma-70 group, σ^{ECF} lacks regions 1 and 3 and instead only harbor the two regions responsible for promoter binding, regions 2 and 4 [22]. Upon analysis, the RpoE structural organization in *Bh* is revealed to belong to the σ^{ECF} family due to the presence of only regions 2 and 4. Based on the genomic organization, the protein can be categorized into the σ^{ECF15} group, where the sigma factor is co-transcribed with its cognate anti-sigma factor, *nepR*, and the *phyR* gene is divergently transcribed [22]. When the amino acid sequence of RpoE is compared to that of other alpha-proteobacteria, the sequence similarity can be observed (the percent identities range from 55.72% to 82.08%) (Fig. 3b). Due to a similar system previously described in *B. quintana* and that the PhyR and RpoE proteins have been confirmed to be homologous to those of other alpha-proteobacterial species, it is reasonable to state that the GSR system is also present in *Bh*.

3.4 *Bh* GSR system is auto-regulated by RpoE and PhyR

It was previously described that the GSR genes auto-regulate their own transcription in other alpha-proteobacteria [22, 37, 38]. We wanted to determine whether this is also the case in *Bh* by testing whether the RpoE and PhyR proteins will affect the activities of the *nepR* and *phyR* promoters upstream of the divergent operons. We constructed nonpolar in-frame deletion constructs of the *rpoE* and *phyR* genes as described in subsection 2.2 of Materials and Methods. We were unable to obtain a deletion mutant for *nepR* despite multiple attempts. This result was not surprising as *nepR* has been shown to be essential in other alphaproteobacteria and thus it would not be possible to delete this gene [12, 37]. We electroporated pNS2 plasmids harboring a *lacZ* reporter gene that is driven by either a *nepR* or *phyR* promoter and performed β -gal assays to determine the strength of the promoters in driving *lacZ* transcription. The transcription of *lacZ* should affect the synthesis of β -gal enzymes and thus its activity should be proportional to the hydrolysis of the enzyme substrate, ONPG. We would expect that once the genes are deleted from the chromosome, the *nepR* and *phyR* promoters would have decreased activity as σ^{EcfG} and PhyR are positive regulators of the GSR system in other alpha-proteobacteria [10, 39, 40]. Our results show

that when *rpoE* and *phyR* are removed from the *Bh* genome, their respective promoters have decreased β -gal activity at 37°C compared to our WT strain, which has fully functional RpoE and PhyR proteins (Fig. 4). The results are in agreement with previous reports [5, 6, 10, 12] and we can conclude that both PhyR and RpoE are positive regulators of the GSR system in *Bh*.

3.5 Transcription of the GSR genes is up-regulated in conditions mimicking the cat flea vector

In order to determine whether the GSR genes of *Bh* are required to adapt to the conditions that mimic the arthropod vector [9], we incubated the bacteria with hemin under temperatures mimicking that of the cat flea (5 mM hemin at 28°C) and the vertebrate host (0.05 mM hemin at 37°C) and analyzed the transcription levels of the GSR genes (Fig. 5). We found that under the conditions mimicking the cat flea vector, the GSR genes are up-regulated compared to conditions mimicking the vertebrate host, indicating that heme is one of the factors that can activate this system in *Bh*. Due to the difference in RCN values for all GSR genes, we cannot rule out the possibility of an alternate promoter in addition to the ECF promoter driving transcription of the GSR genes. In addition, *nepR* has the lowest RCN value and therefore is less transcribed than the other genes. It may be possible that *nepR* is naturally lowly transcribed since it only functions under certain conditions and negatively regulates the GSR system. We also found that heme does not affect transcription of the HWE_HK histidine kinase. The presence of transcriptional terminators or mRNA secondary structure may result in incomplete transcripts wherein the HWE_HK is not detected. Additionally, it may also be possible that the differences observed in RCN may be a result of variable PCR efficiency related to each specific primer pair. While discrepancies were observed in our qRT-PCR results of the GSR genes that were not consistent with the presence of only two divergent mRNA species, a search of the literature did not reveal studies that examine transcription of all the genes in the GSR locus in the same experiment and it is unclear if this phenomenon is observed in other alphaproteobacteria. However, we cannot eliminate the possibility of the existence of multiple polycistronic transcripts that contain one or more GSR genes. In the RT-PCR results (Fig. 2), we identified another potential polycistronic transcript upon which *phyR* and *nepR* are found, leading to the possibility that *phyR* maybe transcribed on more than one transcript. We found that *phyR* is found on separate polycistronic transcripts with both the HWE_HK gene in one case and *nepR* in the other, which would provide a possible explanation for the high transcription of *phyR* while both *nepR* and HWE_HK transcription are much lower. Further examination of the GSR locus is needed to identify potential promoters within the intergenic regions between the GSR genes. It may be possible that such promoters are σ^{EcfG} independent.

3.6 Differential transcription of *badA* and genes encoding heme-binding proteins modulated by the GSR system in *Bh*

BadA is an adhesin responsible for interaction with endothelial cells in the human host, making the protein an important virulence factor during *Bh* pathogenesis [41]. We wanted to determine whether the GSR system would affect the expression of *badA* under various hemin concentrations and different biologically relevant temperatures (cat flea vectors have a cooler internal temperature at 28°C compared to the higher body temperature at 37°C in

the vertebrate host) [42]. The qRT-PCR results reveal that *badA* transcription is increased when the *rpoE* gene is deleted from the *Bh* genome at both temperatures. However, transcription of *badA* is higher at 37°C than at 28°C, suggesting that at the temperature of the vertebrate host, the GSR negatively regulates BadA (Fig. 6). This negative regulation of *badA* by RpoE can be due to the tradeoff between survival and virulence. When the bacteria are under stress, they need the immediate resources to respond to the stress instead of activation of specific virulence factor such as *badA*. It is possible that this regulation may be indirect as we were not able to discern an ECF promoter motif upstream of the *badA* gene in *Bh*. Since our observed results indicate negative regulation of *badA* by *rpoE*, it is tempting to speculate that *rpoE* controls transcription of a repressor that then acts upon *badA*. Thus deletion of *rpoE* would result in down-regulation of the repressor and increased *badA* transcription. The identification of this hypothesized transcriptional repressor would require a detailed analysis of the *Bh* transcriptome to define the GSR regulon.

Bh is a vector-borne pathogen whose vertebrate hosts include the domestic cat reservoir and the incidental human host. The arthropod vector responsible for transmitting the bacteria between cats is the cat flea vector [20]. It has been described that heme concentration is one of the noticeable differences between the cat flea vector and the vertebrate host, with the heme concentration in the cat flea vector being much more toxic than in the vertebrate host [43]. This toxic concentration can be detrimental to most species of bacteria, but *Bh* has retained the ability to survive in this unfavorable condition. One of the mechanisms that the bacteria can potentially use to prevent the toxic heme from destroying the cells is via regulation of stress response by the GSR system.

Several genes encoding heme-binding proteins (Hbps) (A, B, C, and D) are found on the *Bh* genome, and these proteins represent one of the mechanisms that bacteria can use to combat the toxic effects of heme [43]. Since Hbps mediate the binding of toxic heme that can be harmful to the bacteria, they can potentially be regulated by the GSR system. Upon qRT-PCR analysis of the four *hbp* genes in *Bh*, we found that RpoE positively regulates *hbpA* and *hbpC* at both temperatures, with the differences being more obvious at 28°C (Fig. 7a & 7c). The results indicate that RpoE affects binding of heme by Hbps A and C under temperatures relevant to the cat flea vector. On the other hand, transcription of *hbpB* and *hbpD* is higher in *rpoE* at 37°C, the temperature of the vertebrate host, indicating that these genes are negatively regulated by RpoE under conditions relevant to the vertebrate host (Fig. 7b & 7d). Upon a search for ECF promoter motifs upstream of the *hbp* genes, we did not discern such a motif and therefore we predict that regulation of the *hbp* genes may involve a secondary transcriptional regulator in the pathway. As previously mentioned, transcriptomic data will be required to reveal the regulon of the GSR system and allow us to identify downstream transcriptional regulators that may be responsible for transcription of all four *hbp* genes. Our studies show that the GSR system affects transcription of genes encoding Hbps either positively or negatively in *Bh* depending on the host temperature conditions.

3.7 Regulation of *Bh* infection of endothelial cells by the GSR system

Bh infection in humans results in an intimate interaction of the bacteria with host endothelial cells, causing proliferation of endothelial cells, and leading to tumor-like angiogenic lesions

in immunocompromised patients. It is possible that the GSR system may be involved in protection of *Bh* against stresses encountered in the human body during the initial infection. In order to address this question, we infected endothelial cells with the wild-type strain and the *phyR* mutant for two hours and enumerated intracellular bacteria by determining the colony forming units (CFU) by plating of endothelial cell lysates. Unexpectedly, we observed that *Bh* with the mutant *phyR* genotype was more efficient at attaching and invading HMEC-1 cells compared to the wild-type (Fig. 8). These results follow the trend of *badA* expression with the increased *badA* expression observed in the *rpoE* mutant (Fig. 6) correlating with the increase in intracellular bacteria noted in the *phyR* mutant (Fig. 8). BadA is known to be a major virulence factor associated with interaction with host cells [41]. Both PhyR and RpoE were shown to be positive regulators of the GSR system in *Bh*, thus we would expect that they would share a similar regulon resulting in a phenocopy type outcome. Therefore, it is possible that there is a direct correlation between *badA* expression and efficiency of endothelial cell invasion in which the GSR-mediated negative regulation of *badA* would affect the ability of *Bh* to interact with the host cells and render the bacteria less virulent.

3.8 BH13820 (HisKA_2 domain) is a positive regulator of the GSR system in *Bh*

While the interaction between NepR with PhyR and σ^{EcfG} has been well studied, there is still a lack of information regarding the role of histidine kinases associated with the GSR system. In *Bh*, two genes encoding histidine kinases that harbor domains unlike those of typical histidine kinases, e.g. HisKA_2 and HWE_HK, are located downstream of both *rpoE* and *phyR*, respectively. These histidine kinases have previously been shown to regulate the GSR system in other alpha-proteobacterial species either via phosphorylation or dephosphorylation. While the GSR system has been reported in one of the *Bartonella* species, the role of histidine kinases has not been elucidated. In this study, a HisKA_2 deletion mutant was used to determine promoter activity. The *nepR* and *phyR* promoter activities are decreased in the HisKA2_HK histidine kinase mutant compared to WT (Fig. 9). These results indicate that the histidine kinase harboring a HisKA_2 domain is a positive regulator of the GSR system in *Bh* and could potentially function as a kinase by phosphorylating PhyR.

Furthermore, despite multiple attempts we were unable to electroporate either of the promoter plasmids into the HWE_HK histidine kinase deletion mutant. The reasons for this are unclear and do not appear to be technical since we had no difficulty introducing these plasmids into *phyR*, *rpoE* and HisKA_2 histidine kinase deletion mutants. It may be possible that this histidine kinase plays a role regulating genes involved in plasmid replication or stability. Regardless, since it was shown that heme does not affect transcription of this gene (Fig. 5), it is possible that this histidine kinase does not play a role in activation of the GSR.

4. Conclusions

In this study, we identified a GSR system in *Bh* and that the bacterium retains the conserved gene synteny and the PhyR anti-anti-sigma factor and the ECF sigma factor (RpoE). Furthermore, we showed that the *Bh* GSR genes are transcribed on at least two mRNA

transcripts. In contrast to our expectations, we also found that *phyR* and *nepR* may be transcribed on the same transcript due to the presence of an amplification product resulting from primers that targeted the intergenic region between these two genes suggesting the presence of a third mRNA species. In addition, we also showed that both PhyR and RpoE are positive regulators of the GSR system in *Bh*. We also showed that the GSR system is required for adaption to conditions mimicking the cat flea vector and that this system also affects transcription of *hbp* genes and thus regulates the response to heme stress. In addition, the GSR system in *Bh* also regulates transcription of *badA* in conditions that are relevant to the vertebrate host, and thus potentially affects the virulence and host cell interaction of the bacterium. Our infection data followed the same trend as the *badA* expression. Since BadA is a known virulence factor involved in interaction with host cells, it is reasonable to conclude that if the expression of *badA* is repressed by the GSR system, then the ability of the *Bh* to infect endothelial cells would be affected in the same manner. Finally, we also examined the role of histidine kinases in the GSR system of *Bh* and identified a histidine kinase (BH13820) that can potentially phosphorylate PhyR and therefore is a positive regulator of the GSR system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by NIH Grant 1R21AI102560-01A1 to BA.

Role of the funding source

The study was financially supported by NIH/NIAID. This organization was not involved in the study design, the collection, analysis, and interpretation of data. The written report of the study and the decision to submit the article for publication was discussed and decided upon by the authors without any influence from the funding organization.

References

1. Marles-Wright J, Lewis RJ. Stress responses of bacteria. *Curr Opin Struct Biol.* 2007; 17:755–760. [PubMed: 17920859]
2. Storz, G.; Hengge, R. American Society for M. Bacterial stress responses. Washington, DC: ASM Press; 2011.
3. Battesti A, Majdalani N, Gottesman S. The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol.* 2011; 65:189–213. [PubMed: 21639793]
4. Hecker M, Pané-Farré J, Uwe V. SigB-Dependent General Stress Response in *Bacillus subtilis* and Related Gram-Positive Bacteria. *Annu Rev Microbiol.* 2007; 61:215–236. [PubMed: 18035607]
5. Francez-Charlot A, Kaczmarczyk A, Fischer H-M, Vorholt JA. The general stress response in Alphaproteobacteria. *Trends Microbiol.* 2015; 23:164–171. [PubMed: 25582885]
6. Staron A, Mascher T. General stress response in alpha-proteobacteria: PhyR and beyond. *Mol Microbiol.* 2010; 78:271–277. [PubMed: 20979331]
7. Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. *Annu Rev Biochem.* 2000; 69:183–215. [PubMed: 10966457]
8. Francez-Charlot A, Frunzke J, Reichen C, Ebnetter JZ, Gourion B, Vorholt JA. Sigma factor mimicry involved in regulation of general stress response. *P Natl Acad Sci USA.* 2009; 106:3467–3472.

9. Abromaitis S, Koehler JE. The *Bartonella quintana* extracytoplasmic function sigma factor RpoE has a role in bacterial adaptation to the arthropod vector environment. *J Bacteriol.* 2013; 195:2662–2674. [PubMed: 23564167]
10. Gourion B, Sulser S, Frunzke J, Francez-Charlot A, Stiefel P, Pessi G, et al. The PhyR- σ EcfG signalling cascade is involved in stress response and symbiotic efficiency in *Bradyrhizobium japonicum*. *Mol Microbiol.* 2009; 73:291–305. [PubMed: 19555458]
11. Kim H-S, Caswell CC, Foreman R, Roop RM, Crosson S. The *Brucella abortus* General Stress Response System Regulates Chronic Mammalian Infection and Is Controlled by Phosphorylation and Proteolysis. *J Biol Chem.* 2013; 288:13906–13916. [PubMed: 23546883]
12. Lourenço RF, Kohler C, Gomes SL. A two-component system, an anti-sigma factor and two paralogous ECF sigma factors are involved in the control of general stress response in *Caulobacter crescentus*. *Mol Microbiol.* 2011; 80:1598–1612. [PubMed: 21564331]
13. Jans A, Vercruysse M, Gao S, Engelen K, Lambrechts I, Fauvart M, et al. Canonical and non-canonical EcfG sigma factors control the general stress response in *Rhizobium etli*. *MicrobiologyOpen.* 2013; 2:976–987. [PubMed: 24311555]
14. Kaczmarczyk A, Campagne S, Danza F, Metzger LC, Vorholt JA, Francez-Charlot A. Role of *Sphingomonas* sp. Strain Fr1 PhyR-NepR- σ EcfG Cascade in General Stress Response and Identification of a Negative Regulator of PhyR. *J Bacteriol.* 2011; 193:6629–6638. [PubMed: 21949070]
15. Kim H-S, Willett JW, Jain-Gupta N, Fiebig A, Crosson S. The *Brucella abortus* virulence regulator, LovhK, is a sensor kinase in the general stress response signalling pathway. *Mol Microbiol.* 2014; 94:913–925. [PubMed: 25257300]
16. Sauviac L, Bruand C. A Putative Bifunctional Histidine Kinase/Phosphatase of the HWE Family Exerts Positive and Negative Control on the *Sinorhizobium meliloti* General Stress Response. *J Bacteriol.* 2014; 196:2526–2535. [PubMed: 24794560]
17. Anderson BE, Neuman MA. *Bartonella* spp. as emerging human pathogens. *Clin Microbiol Rev.* 1997; 10:203–219. [PubMed: 9105751]
18. Eicher SC, Dehio C. *Bartonella* entry mechanisms into mammalian host cells. *Cell Microbiol.* 2012; 14:1166–1173. [PubMed: 22519749]
19. Kaiser PO, Riess T, O'Rourke F, Linke D, Kempf VA. *Bartonella* spp.: throwing light on uncommon human infections. *Int J Med Microbiol.* 2011; 301:7–15. [PubMed: 20833105]
20. Dehio C. *Bartonella*-host-cell interactions and vascular tumour formation. *Nature Rev Microbiol.* 2005; 3:621–631. [PubMed: 16064054]
21. Resto-Ruiz SI, Sweger D, Widen RH, Valkov N, Anderson BE. Transcriptional activation of the *htrA* (High-temperature requirement A) gene from *Bartonella henselae*. *Infect Immun.* 2000; 68:5970–5978. [PubMed: 10992509]
22. Staron A, Sofia HJ, Dietrich S, Ulrich LE, Liesegang H, Mascher T. The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. *Mol Microbiol.* 2009; 74:557–581. [PubMed: 19737356]
23. Regnery RL, Anderson BE, Clarridge JE III, Rodriguez-Barradas M, Jones DC, Carr JH. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. *J Clin Microbiol.* 1992; 30:265–274. [PubMed: 1371515]
24. Riess T, Dietrich F, Schmidt KV, Kaiser PO, Schwarz H, Schafer A, et al. Analysis of a novel insect cell culture medium-based growth medium for *Bartonella* species. *Appl Environ Microbiol.* 2008; 74:5224–5227. [PubMed: 18567689]
25. Gillaspie D, Perkins I, Larsen K, McCord A, Pangonis S, Sweger D, et al. Plasmid-based system for high-level gene expression and antisense gene knockdown in *Bartonella henselae*. *Appl Environ Microbiol.* 2009; 75:5434–5436. [PubMed: 19542333]
26. Mackichan JK, Gerns HL, Chen YT, Zhang P, Koehler JE. A SacB Mutagenesis Strategy Reveals that the *Bartonella quintana* Variably-Expressed Outer Membrane Proteins (Vomp) are Required for Bloodstream Infection of the Host. *Infect Immun.* 2007
27. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. *Nucleic Acids Res.* 2015; 43:D222–D226. [PubMed: 25414356]

28. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, et al. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* 2011; 39:D225–D229. [PubMed: 21109532]
29. Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, et al. CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res.* 2009; 37:D205–D210. [PubMed: 18984618]
30. Marchler-Bauer A, Bryant SH. CD-Search: protein domain annotations on the fly. *Nucleic Acids Res.* 2004; 32:W327–W331. [PubMed: 15215404]
31. Pulliainen AT, Dehio C. Persistence of *Bartonella* spp. stealth pathogens: from subclinical infections to vasoproliferative tumor formation. *FEMS Microbiol Rev.* 2012; 36:563–599. [PubMed: 22229763]
32. Windholz, M. The Merck index : an encyclopaedia of chemicals, drugs, and biologicals. Rahway: Merck;
33. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc.* 2008; 3:1101–1108. [PubMed: 18546601]
34. Miller, J. Experiments in Molecular Genetics. Cold Spring Harbor, NY: CSH Laboratory Press; 1972.
35. McCord AM, Resto-Ruiz SI, Anderson BE. Autocrine role for interleukin-8 in *Bartonella henselae*-induced angiogenesis. *Infect Immun.* 2006; 74:5185–5190. [PubMed: 16926411]
36. Foreman R, Fiebig A, Crosson S. The LovK-LovR Two-Component System Is a Regulator of the General Stress Pathway in *Caulobacter crescentus*. *J Bacteriol.* 2012; 194:3038–3049. [PubMed: 22408156]
37. Sauviac L, Philippe H, Phok K, Bruand C. An Extracytoplasmic Function Sigma Factor Acts as a General Stress Response Regulator in *Sinorhizobium meliloti*. *J Bacteriol.* 2007; 189:4204–4216. [PubMed: 17400745]
38. Alvarez-Martinez CE, Lourenço RF, Baldini RL, Laub MT, Gomes SL. The ECF sigma factor σ^T is involved in osmotic and oxidative stress responses in *Caulobacter crescentus*. *Mol Microbiol.* 2007; 66:1240–1255. [PubMed: 17986185]
39. Bastiat B, Sauviac L, Bruand C. Dual Control of *Sinorhizobium meliloti* RpoE2 Sigma Factor Activity by Two PhyR-Type Two-Component Response Regulators. *J Bacteriol.* 2010; 192:2255–2265. [PubMed: 20154128]
40. Herrou J, Foreman R, Fiebig A, Crosson S. A structural model of anti-anti-sigma inhibition by a two-component receiver domain: the PhyR stress response regulator. *Mol Microbiol.* 2010; 78:290–304. [PubMed: 20735776]
41. Riess T, Andersson SG, Lupas A, Schaller M, Schafer A, Kyme P, et al. *Bartonella* adhesin a mediates a proangiogenic host cell response. *J Exp Med.* 2004; 200:1267–1278. [PubMed: 15534369]
42. Liu M, Ferrandez Y, Bouhsira E, Monteil M, Franc M, Boulouis H-J, et al. Heme Binding Proteins of *Bartonella henselae* Are Required when Undergoing Oxidative Stress During Cell and Flea Invasion. *PLoS ONE.* 2012; 7:e48408. [PubMed: 23144761]
43. Graça-Souza AV, Maya-Monteiro C, Paiva-Silva GO, Braz GRC, Paes MC, Sorgine MHF, et al. Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochem Mol Biol.* 2006; 36:322–335. [PubMed: 16551546]

Highlights

- The GSR was characterized in the alpha-proteobacterium *Bartonella henselae*.
- The GSR adapts gene expression patterns in conditions mimicking the flea vector
- The GSR modulates expression of virulence and heme-binding genes of *B. henselae*
- A histidine kinase (BH13820) positively regulates the GSR in *B. henselae*

Figure 1. Gene synteny is conserved in *Bh* and other alpha-proteobacteria

The gene arrangement in the *Bh* GSR locus shows that *phyR* and *nepR* are divergently arranged and the gene encoding σ^{EcfG} is located downstream of *nepR*. In some alpha-proteobacteria, genes encoding histidine kinases are also located in the gene cluster. In *Bh*, two histidine kinase genes are located both downstream of *rpoE* and *phyR*. Abbreviation of species name: *Bartonella henselae* (*Bh*), *Bartonella quintana* (*Bq*), *Brucella abortus* (*Ba*), *Bradyrhizobium diazoefficiens* (*Bd*), *Caulobacter crescentus* (*Cc*), *Methylobacterium extorquens* (*Me*), *Rhizobium etli* (*Re*), *Rhodopseudomonas palustris* (*Rp*), *Sinorhizobium meliloti* (*Sm*).

Figure 2. Transcription of the GSR genes in *Bh*

The RNA was extracted from *Bh*, treated with DNase, and converted into cDNA using reverse transcriptase. For the PCR, different templates include *Bh* RNA with reverse transcriptase (lane 1), *Bh* RNA without reverse transcriptase (lane 2), water only (lane 3) and genomic DNA from *Bh* Houston-1 (lane 4).

Figure 3. GSR PhyR and σ^{EcfG} are conserved in *Bh*

Bh PhyR and RpoE amino acid sequences were aligned with those of other alpha-proteobacterial species and percent identities were calculated based on the sequence alignment. The heat map shows the amount of conservation throughout the amino acid sequences among the alpha-proteobacterial species. The degree of conservation is represented by the black and white shading of the heat map. Black areas indicate the highest conservation and white areas indicate the lowest conservation. Grey areas represent regions with intermediate conservation. **A)** PhyR share sequence similarity with other PhyR as well as similar domain structures. *Bh* PhyR contains a receiver (REC) domain at the C-terminus and the N-terminus of PhyR contains a sigma factor-like domain. **B)** RpoE in *Bh* belongs to the σ^{ECF} sub-group, as is the case of the alternate sigma factor involved in the GSR system of other alpha-proteobacterial species. *Bh* RpoE contains only regions 2 and 4 and lacks regions 1 and 3, which is characteristic of σ^{ECF} structural organization. Furthermore, *Bh* RpoE shares amino acid sequence similarity with other alphaproteobacterial GSR ECF sigma factor proteins.

Figure 4. RpoE and PhyR auto-regulates expression of GSR genes

The activities of the *nepR* (A) and *phyR* (B) promoters were analyzed by measuring the enzymatic hydrolysis of ONPG by β -gal in different deletion mutants of the *rpoE* and *phyR* genes compared to the wild-type (WT). Both the *nepR* and *phyR* promoters have decreased β -gal activity in both *rpoE* and *phyR* compared to WT at 37°C.

Figure 5. Transcription of GSR genes is increased under conditions mimicking the cat flea vector WT bacteria were grown in Schneider's medium and exposed to either 5 mM hemin at 28°C or 0.05 mM hemin at 37°C. Total RNA was extracted, treated with DNase, and converted into cDNA, and qRT-PCR was performed. Transcription of each GSR gene, except HWE_HK, is increased at 5 mM at 28°C, the condition that mimics the cat flea vector.

Figure 6. Transcription of *badA* in *rpoE* compared to the wild-type (WT) strain

Transcript levels of *badA* was determined by qRT-PCR and was observed to be higher in *rpoE* compared to WT at both temperature conditions, although *badA* mRNA levels are higher at 37°C compared to 28°C. Higher transcription of *badA* in *rpoE* suggests that this sigma factor negatively regulates transcription of *badA*.

Figure 7. Differential transcription of genes encoding Hbps

Bacteria were grown in 1 mM hemin and incubated at either 28°C or 37°C [9]. qRT-PCR of the genes encoding Hbps A (a), B (b), C (c), and D (d) were performed.

Figure 8. Regulation of endothelial cell infection by the GSR system in *Bh*
Bh were used to infect HMEC-1 endothelial cells at a multiplicity of infection of 100. The infection was carried out for two hours, then the bacteria were plated onto chocolate plates and colonies were counted following 7 days of incubation at 37°C, 5% CO₂.

Figure 9. BH13820 (HisKA_2) positively regulates the GSR system

nepR and *phyR* promoter activities are decreased when the gene is deleted from the genome, indicating that BH13820 is a potential kinase that phosphorylates PhyR.