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Characterization of the general stress response in *Bartonella henselae*

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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.

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 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

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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 *Bartonella quintana* and shown to be involved in the adaptation of this bacterium to the lower temperature and higher hemin 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% CO_2 [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* (BH138550) 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 megaprime 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