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A dual component heme biosensor that integrates heme transport and synthesis in bacteria

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ABSTRACT

Bacterial pathogens acquire host iron to power cellular processes and replication. Heme, an iron-containing cofactor bound to hemoglobin, is scavenged by bacterial proteins to attain iron. Methods to measure intracellular heme are laborious, involve complex chemistry, or require radioactivity. Such drawbacks limit the study of the mechanistic steps of heme transport and breakdown. Hypothesizing heme homeostasis could be measured with fluorescent methods, we coupled the conversion of heme to biliverdin IXα (a product of heme catabolism) by heme oxygenase 1 (HO1) with the production of near-infrared light upon binding this verdin by infrared fluorescent protein (IFP1.4). The resultant heme sensor, IFP-HO1, was fluorescent in pathogenic *E. coli* exposed to heme but not in the absence of the heme transporter ChuA and membrane coupling protein TonB, thereby validating their long-standing proposed role in heme uptake. Fluorescence was abolished in a strain lacking *hemE*, the central gene in the heme biosynthetic pathway, but stimulated by iron, signifying the sensor reports on intracellular heme production. Finally, an invasive strain of *E. coli* harboring the sensor was fluorescent during an active infection. This work will allow researchers to expand the molecular toolbox used to study heme and iron acquisition in culture and during infection.

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1. Introduction

The ability of a bacterium to replicate is dependent on a number of factors, including being able to attain sufficient amounts of critical metals, including iron (Heinemann et al., 2008; Nobles and Maresso, 2011; Skaar, 2010). This includes pathogenic bacteria that possess multiple iron acquisition systems adapted to host niches such as the gastrointestinal tract and blood (Nobles and Maresso, 2011; Runyen-Janecky, 2013; Skaar, 2010). The host counters this response with a growth-restricting strategy termed nutritional immunity (Hood and Skaar, 2012). The most abundant iron reservoir in mammals is heme, an iron–porphyrin cofactor bound to hemoglobin that supports the transport of oxygen to the body's cells (Nand, 2003, pp. 1399–1400). Heme uptake systems have now been identified in many bacterial pathogens, including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus anthracis*, *Yersinia pestis* and *Escherichia coli* (Nobles and Maresso, 2011; Runyen-Janecky, 2013; Ryter and Tyrrell, 2000). Perhaps the most extensively studied of these is the outer membrane receptor ChuA, first described in enterohemorrhagic *E. coli* (EHEC), a prominent cause of diarrhea and kidney nephrotoxicity (Bhunia, 2008; Torres and Payne, 1997). ChuA can bind heme and transport the iron–porphyrin into the periplasm (Runyen-Janecky, 2013; Torres and Payne, 1997). The energy for transport is supplied by the proton gradient across the cytoplasmic membrane through the energy transferring complex TonB–ExhB–ExhD (Krewulak and Vogel, 2011; Torres and Payne, 1997). Once in the cytosol, heme can be degraded by a class of enzymes generally referred to as heme oxygenases. These oxygenases facilitate the oxidative cleavage of the tetrapyrrolic ring by utilizing the iron molecule within the heme to coordinate oxygen. The reaction produces molecular iron, carbon monoxide, and biliverdin (Ryter and Tyrrell, 2000; Wilks and Heinzl, 2014). The first heme oxygenase to be biochemically characterized was Hmox1 (HO1), which utilizes three molecules of oxygen and seven of NADPH with cytochrome P450 NADPH-reductase to catalyze the oxidative cleavage of heme to produce biliverdin Ixα (EC 1.14.99.3). This regiospecificity (production of the α-isoform) is not always shared across bacterial heme oxygenases and several enzymes have been described now that produce other isoforms (Wilks and Heinzl, 2014; Wilks et al., 1995). If heme is low but free iron is present, bacteria such as *E. coli* can also synthesize heme *via* the genes encoding the Hem A–H biosynthetic enzymes. Uroporphyrinogen decarboxylase, encoded by the *hemE* gene, is a key enzyme in the pathway that leads to heme biosynthesis and is required for heme production (Săsărman et al., 1975). Heme synthesized in this manner is incorporated into cytochrome proteins and the electron transport chain to facilitate energy production (Puustinen et al., 1992).

Due to the central importance of heme in the biology of bacterial pathogens, we sought to construct a fluorescence-based system that would conveniently report on heme metabolism without the use of

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cell destroying chemical or radiologic methods. Here, we report the construction of a heme biosensor (IFP-HO1) consisting of infrared fluorescent protein (IFP1.4), engineered from a bacteriophytochrome of *Deinococcus radiodurans*, and human heme oxygenase 1 (HO1). IFP1.4 produces near-infrared light when bound to biliverdin Ixα, which is a product of heme degradation facilitated by HO1 (Shu et al., 2009). We demonstrate proof-of-principle studies using IFP-HO1 to report on bacterial heme intake, processing and biosynthesis, define methods to yield quantifiable outputs for its use, and demonstrate its versatility as a real-time reporter of heme homeostasis during infection of a vertebrate host. Such a sensor can be used to determine the contribution of bacterial genes to heme uptake and metabolism, expand our knowledge of the temporal regulation of these processes, and highlight when and how such systems function during host infection. Since there have also been attempts to develop antimicrobials that inhibit heme or iron transport (Furci et al., 2007; Owens et al., 2013), this tool can also be used to screen for inhibitors of these important bacterial pathways.

2. Materials and methods

2.1. Bacterial strains and reagents

Bacterial strains used in this study include *E. coli* serotype O157:H7 strain EDL933 (ATCC# 700927) and the clinical isolate *E. coli* CP9 (Russo et al., 1996; Torres and Payne, 1997). The plasmids were graciously given by Dr. Alfredo Torres (University of Texas Medical Branch, Galveston, TX) and included pCHU101 (pACYC184-based vector containing ChuA under the native *E. coli* (EDL933) promoter), and pBJM2 (pACYC177-based vector containing TonB under native promotor) (Torres and Payne, 1997). *E. coli* was grown at 37 °C in Lysogeny-broth (LB, iron-rich media), M9 media, or M9 media supplemented with 0.6% CAS amino acids (M9Cas, iron-starved media). Cultures were started from a single colony from LB agar plates using aseptic techniques. Ampicillin (100 μg/mL), kanamycin (25 μg/mL), and chloramphenicol (30 μg/mL) were supplemented into the media when necessary to maintain plasmids. Hemin (heme) was purchased from Sigma Life Science (H9039-100G); biliverdin hydrochloride (biliverdin) from Frontier Scientific (B655-9); ferrous sulfate ($FeSO_4$) from J.T. Baker (2074-01); NADPH-P450 oxidoreductase, recombinant human, from Calbiochem (481974); bovine serum albumin (BSA) from Fisher Scientific (9048-46-8); ampicillin from USB corporation (69-52-3); isopropyl-B-D-thiogalactopyranoside (IPTG) from TEKnova (I3325); Deferoxamine mesylate salt (DFA) from Sigma Life Science (D9533-1G); 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridone (DFP) from Aldrich Chemistry (379409-5G); 2,2-bipyridine (2,2-DP) from Alfa Aesar (366-18-7); and glutathione (GT) from Calbiochem (#3541).

2.2. Construction of mutant strains

PKD46, a plasmid carrying ampicillin resistance and λ red recombinase under an arabinose promoter, was transformed into chemically competent wildtype *E. coli* EDL933 or CP9 and transformants selected on LB plates containing 100 μg/mL ampicillin. Sequences homologous to the flanking region of the gene of interest were amplified using PCR and appropriate primers containing FRT sites. pBA169CM:FRT was used as a template to amplify the flanking regions and FRT sites. The end-product contained the flanking regions adjacent to FRT sites with a chloramphenicol resistance cassette located between them. Purified PCR products were transformed into the EDL933 or CP9 WT containing PKD46 grown in the presence of L-arabinose to induce the expression of λ red recombinase. Successful recombined strains were screened for growth on chloramphenicol and verified by PCR using the forward primer for the upstream region and either a primer specific for the gene of interest or the chloramphenicol resistance cassette. To remove PKD46, recombinant strains were grown overnight at 42 °C. Loss of PKD46 was verified by patch-plating on both ampicillin and chloramphenicol to screen for loss of ampicillin resistance.

2.3. Plasmid construction and protein purification

The *ifp1.4* and *ho1* genes were amplified from a pcDNA3.1-IFP1.4 vector graciously given to our lab by Dr. Hua Chen (Baylor College of Medicine) and from human cDNA (ATCC# 3504480), respectively. The *ifp1.4* gene was initially amplified by PCR using primers to fuse a HA-tag sequence for immunodetection: forward IFP1.4 primer — GATCGA TCGGTACCCCATGGCTCGGGACCCTC, reverse primer — GTTAATATGGTA CCTTATGCATAATCCGGAACATCATACGGATAGGCTTCTTTCCTCTG. Additional primers were used to amplify *ifp1.4-ha* to include restriction sites BamHI and EcoRI: forward primer — GACTGACTGGATCCATGGCT CGGGAC, reverse primer — CCAGAGTTGAATTCGCTGGTACCTTATGC. Primers used to amplify *ho1* shared similar features including the restriction sites and addition of an HA-tag sequence: forward primer — GATCGATCGGATCCATGGAGCGTCCGCAACCC, reverse primer — AATG AATTCTTATTATGCATAATCCGGAACATCATACGGATAAGCCTGGGAGCG GGTGTT. The *ifp1.4-ha* and *ho1-ha* genes were amplified by PCR, cloned into a pGEX2-TK vector, and transformed into DH5α *E. coli* (NEB 5-alpha competent *E. coli*, #C2987I). Following screening and verification of correct constructs, pGEX2-TK-IFP1.4-HA (pGEX-IFP) and pGEX2-TK-HO1-HA (pGEX-HO1) were transformed into a BL21 strain of *E. coli* for protein production (NEB, #C2530H). Expression and purification procedures were similar to previously published methods (Balderas et al., 2012). Proteins were washed and eluted in Tris buffer (50 mM, pH 8.0) and glutathione (6 mM), respectively. Protein samples were dialyzed overnight to remove glutathione against 4 L of Tris buffer. Protein concentration was determined using a Bio-Rad Protein Assay (#500-0006) with BSA as a standard.

Constructs for expressing and purifying IFP1.4 and HO1 were used as templates to PCR amplify HA-tagged genes. Forward primers designed to amplify *ifp1.4-ha* and *ho1-ha* included SalI restriction sites: IFP1.4 forward primer — GCCTGCAGGTCGACTATGGCTCGGGACCCTC, HO1 forward primer — GCCTGCAGGTCGACTATGGAGCGTCCGCAACC. The two genes were PCR amplified with the same reverse primer that included NotI and BamHI sites: reverse primer — GTACCCGGGGATCCGC TAGCTCTAGCGGCCGC TCTATTATTATGCATAATCCGGAACAT. PCR products were cloned into the pUC19 vector (Invitrogen, #54357) using SalI and BamHI restriction sites. Ligations were transformed into DH5α *E. coli* and selected for on LB agar plates with ampicillin. Colonies were selected, screened, and sequenced to verify the construction of pUC19-IFP1.4-HA (pIFP) and pUC19-HO1-HA (pHO1). To construct a pUC19 vector carrying both genes, first *ho1-ha* was PCR amplified using the forward primer GCTCAAGCGGC CGCTGCGTAGCGTAGCGTAG CGAGGAGGTTTATATGGAGCGTCCGCAAC and the reverse primer GGCG ATGGATCCTTATTATGCATAATCCGGAACA TCATACGGATAAGCCTGGGA GCGGG. The PCR product was then digested and ligated into the pIFP1.4 vector at the NotI and BamHI restriction sites. Ligation reactions were then transformed into DH5α *E. coli* and isolated colonies were screened and sequence verified. After verification, plasmid constructs (pUC19, pIFP, pHO1, and pIFP-HO1) were transformed by electroporation into *E. coli* EDL933 or CP9 and selected for on LB agar with ampicillin.

2.4. Bacterial growth and fluorescence assays

Bacterial growth and fluorescence measurements were conducted using a BioTek Synergy HT plate reader (units designated as RFU) or a Tecan Infinite M200 Pro plate reader (units designated as RLU_t). To quantify the fluorescence intensity, the BioTek plate reader was equipped with an excitation filter at 645 nm with a 40 nm bandwidth and an emission filter of 710 nm (20 nm bandwidth), while the Tecan plate reader was set to read excitation at 680 nm (9 nm bandwidth) and emission 710 nm (20 nm bandwidth). Cultures were started from

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