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PII: S0167-7012(15)30020-8
DOI: doi: [10.1016/j.mimet.2015.07.011](https://doi.org/10.1016/j.mimet.2015.07.011)
Reference: MIMET 4691

To appear in: *Journal of Microbiological Methods*

Received date: 11 May 2015
Revised date: 9 July 2015
Accepted date: 9 July 2015



Please cite this article as: Nobles, Christopher L., Clark, Justin R., Green, Sabrina I., Maresso, Anthony W., A dual component heme biosensor that integrates heme transport and synthesis in bacteria, *Journal of Microbiological Methods* (2015), doi: [10.1016/j.mimet.2015.07.011](https://doi.org/10.1016/j.mimet.2015.07.011)

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Title Page

**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 Ix α (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.

Keywords: Heme; Iron; IFP1.4; *Escherichia coli* O157:H7; biosensor; pathogenesis

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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

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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 Ix α (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 (Săsarman 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 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 Ix α , which is a product of heme degradation facilitated by HO1

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(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.

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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 promoter) (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 sulphate (FeSO₄) 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).

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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 μ g/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

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PCR using primers to fuse a HA-tag sequence for immunodetection: forward IFP1.4 primer – GATCGATCGGTACCCCATGGCTCGGGACCCTC, reverse primer – GTTAATATGGTACCTTATGCATAATCCGGAACATCATACGGATAGGCTTCTTTCCTC TG. Additional primers were used to amplify *ifp1.4-ha* to include restriction sites BamHI and EcoRI: forward primer – GACTGACTGGATCCATGGCTCGGGAC, 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 – AATGAATTCTTATTATGCATAATCCGGAACATCATACGGATAAGCCTGGGAGCGGG TGTT. The *ifp1.4-ha* and *ho1-ha* genes were amplified by PCR, cloned into a pGEX2-TK vector, and transformed into DH5 α *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 Sall restriction sites: IFP1.4 forward primer -

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GCCTGCAGGTCGACTATGGCTCGGGACCCTC, HO1 forward primer -

GCCTGCAGGTCGACTATGGAGCGTCCGCAACC. The two genes were PCR amplified with the same reverse primer that included NotI and BamHI sites: reverse primer – GTACCCGGGGATCCGCTAGCTCTAGCGGCCGC 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 CGCTGCGTAGCGTAGCGTAGCGAGGAGGTTTATATGGAGCGTCCGCAAC and the reverse primer GGCGATGGATCCTTATTATGCATAATCCGGAACA TCATACGGATAAGCCTGGGAGCGGG. 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

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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 single colonies incubated overnight in LB media with selective antibiotics. Prior to experiments, overnight cultures were subsequently subcultured at a 1:100 dilution into fresh LB media with antibiotic. After two hours (approximately mid-log phase), bacteria were isolated and washed with M9 media to remove any residual LB. *E. coli* strains were inoculated into experimental conditions at a final OD_{600} of 0.1 (1 cm pathlength, Beckman Coulter DU 800) in final conditions specified within each experiment. Culturing conditions, whether overnight, subcultures, or experiments, were consistently 37°C while shaking. Growth and fluorescence experiments conducted with the BioTek plate reader were measured every 15 minutes for OD_{600} and fluorescence at 710 nm.

2.5 Heme binding and degradation

Purified HO1 protein (2.5 μ M) was incubated with various concentrations of heme for 30 minutes at 37°C in a crystal cuvette. To determine heme degradation, mixtures with or without heme, purified HO1 (1.0 μ M), and ascorbic acid (500 μ M) were incubated for 30 minutes at 37°C. The absorbance at 400 nm was then measured for each reaction. Where necessary, IFP1.4 was added for 2 hours at 37°C with or without heme (10 μ M), biliverdin (10 μ M), HO1 (0.5 μ M), cytochrome p450 (0.75 μ M), NADPH

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(200 μ M), and BSA (100 ng/mL) (Griffith and Wolf, 2002; Wilks et al., 1995) and the fluorescence recorded at 700 nm. Since fluorescent signal can be used as a direct quantitative output, IFP-HO1 was used to determine the amount of heme converted to biliverdin in intact bacterial cells since the reactant and product are stoichiometric with each other (Shu et al., 2009). This was achieved by experimentally determining two key values, the apparent dissociation constant (k_{d-app}) of IFP for biliverdin and the maximum fluorescence possible at any given density of *E. coli* (RLU_{max}). Once these two values are calculated, one can determine the concentration of biliverdin that yielded a measured and known fluorescence of light by simply solving for [BV] in Equations 1 and 2 (below). To determine the relationship between a measured fluorescence output and the concentration of biliverdin, Equation 1 was used: (1) $RLU = \frac{[BV] * RLU_{max}}{k_{d-app} + [BV]}$.

After measuring the k_{d-app} of biliverdin binding to IFP and the maximum fluorescence possible at any given density of *E. coli* (RLU_{max}), Equation 1 was solved for [BV] to give

Equation 2: $[BV] = \frac{RLU * 65}{7393 * OD600 - RLU}$. The k_{d-app} was measured by titrating biliverdin

into a known amount of purified IFP in PBS (Fig. S1A). This yielded a value of 65 ± 9 nM. The maximum fluorescence (RLU_{max}) was determined by adding biliverdin to lysates attained from mid-log phase *E. coli* expressing IFP (Fig S1B). Using Equation 2, the amount of biliverdin generated by cells expressing IFP-HO can be determined experimentally in real-time using a microplate reader capable of measuring the fluorescence at any given optical density (600 nm). Applying this equation to the data presented in Fig. 3B, an approximate amount of biliverdin produced during the peak

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level of fluorescence (~2-3 hrs) was calculated, yielding estimates of 60 nM (2 hrs), 120 nM (2.5 hrs), and 90 nM (3 hrs – Fig S1C). Using this approach, an approximation of the amount of biliverdin generated can be made by knowledge of the optical density and fluorescence of *E. coli* expressing IFP-HO1. This amount of biliverdin can then be used to estimate the amount of heme that was converted to biliverdin, assuming a 1:1 stoichiometry between the reactant and the biliverdin product.

2.6 The relationship between IFP fluorescence and heme levels and determination of the IFP half-life

Purified IFP1.4 (1.0 μ M) was incubated in PBS in a 96-well plate with increasing concentrations of biliverdin (0.01-5,000 μ M) at 37°C shaking in the BioTek plate reader. Fluorescence was read at two minute intervals until equilibrium. Dissociation constant and maximum fluorescence was obtained by curve fitting with a One-Site total binding analysis in GraphPad Prism version 6 for Windows, GraphPad Software, San Diego, California USA. For the IFP half-life measurements, EDL933 strains containing either pIFP-HO1 or pHO1 were grown as previously stated. At appropriate time-points (2, 3.5, 5, and 7 hours) the protein translation inhibitor kanamycin was added to a final concentration of 250 μ g/ml. To verify that kanamycin was having the intended effect of preventing further growth of the cells, the cultures were monitored for a lack of change in OD600 (Lehtinen et al., 2006). Loss of fluorescence after the addition of kanamycin was standardized and fit to an exponential decay curve using GraphPad Prism version 6.

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2.7 Animal Infections

Extraintestinal pathogenic *E. coli* (ExPEC) CP9 carrying either the pUC19 IFP-HO1 construct or the empty vector were grown overnight in LB containing ampicillin. The following day the cultures were back-diluted 1:100 in fresh LB-amp and grown with shaking at 37°C until mid-log phase (OD₆₀₀ of 0.5-0.8). Cultures were then pelleted and resuspended in PBS to an OD₆₀₀ of 1, corresponding to 10⁹ cfu/ml. Seven week old C57BL/6 mice were next injected intraperitoneally (for organ harvests) or subcutaneously (for granuloma formation) with 100 µl (10⁸ cfu) of CP9 carrying IFP-HO1 or the vector control. Mice also received a subcutaneous injection of 200 µg/g body weight of ampicillin to promote the maintenance of the plasmid. To induce expression of IFP-HO1, mice were given water containing 10 mM IPTG, which has been shown to be sufficient for de-repression of the *lac* promoter in *in vivo* (Cronin et al., 2001). Mice were sacrificed after 24 hours, the organs (or granuloma) harvested by necropsy, and each organ analyzed for fluorescence using a Li-Cor Odyssey Classic® Infrared scanner (IR scans were performed on whole organs held in a 6 well plate). IR intensity was analyzed using Li-Cor Image Studio Lite 4.0 and mean intensity reported. Organs were then homogenized and plated on LB plates containing 100 µg/mL ampicillin to determine the numbers of cfu/g tissue

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3. Results

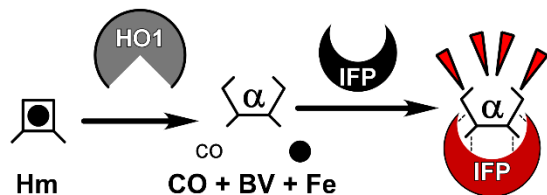
3.1 Construction of a heme biosensor

We reasoned that coupling human heme oxygenase 1 (HO1), which generates biliverdin IX α from heme, with IFP1.4, a bacterial phytochrome protein that emits near-infrared light upon binding biliverdin IX α , would yield a system capable of a convenient readout of heme homeostasis in bacterial pathogens (Fig. 1A) (Shu et al., 2009). We first cloned and purified recombinant IFP1.4 and HO1 similar to previously established methods (Shu et al., 2009; Wilks et al., 1995). Recombinant HO1 functioned as expected with a dose-dependent increase in the Soret absorbance (an indicator of bound heme) upon the mixing of HO1 and heme (Fig. 1B) and demonstrated a decrease in heme when ascorbate, a source of electrons, was added (Fig. S2). Similarly, recombinant IFP, when mixed with biliverdin IX α , produced a dose-dependent increase in fluorescence that saturated near a 1:1 molar ratio of IFP to biliverdin IX α , suggesting the binding was stoichiometric (Fig. 1C). To determine if both reactions (i.e. HO1-mediated conversion of heme to biliverdin and the biliverdin-dependent production of light from IFP) could be coupled to yield a two-system sensor of heme processing, we mixed heme, ascorbate (or NAPH where indicated), HO1, and IFP and measured the fluorescence that results. As shown in Fig. 1D, this combination of reagents led to a fluorescence level that was at least one order of magnitude greater than control reactions made of each individual component, or, on average, 4-8 fold greater than reactions that contained every component but one. When taken together, these results

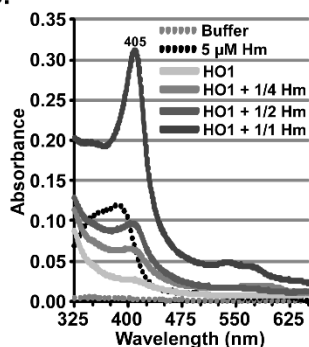
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suggest a heme sensor system can be generated that provides a fluorescent readout of the state of heme under a controlled set of conditions.

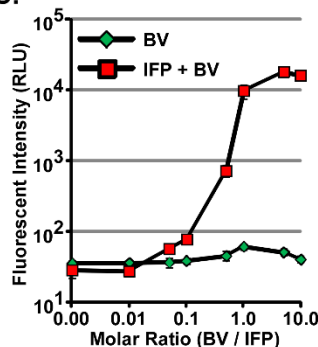
A.



B.



C.



D.

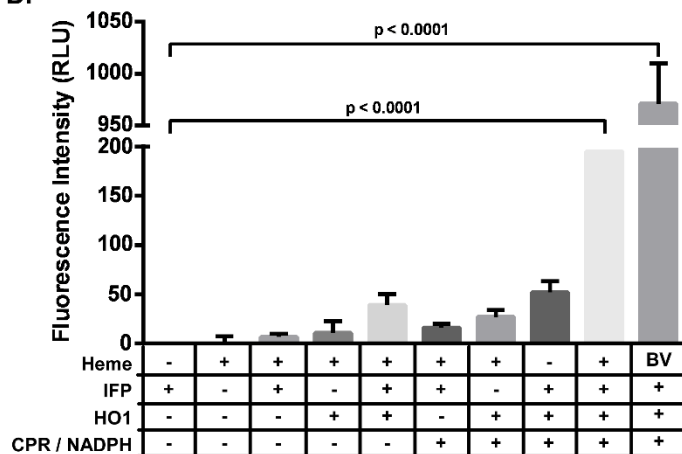


Figure 1. The construction of a heme biosensor using HO1 and IFP1.4. (A) Heme (Hm) can be oxidatively degraded by HO1 to produce the products of carbon monoxide (CO), biliverdin IX α (BV), and iron (Fe). Biliverdin, when bound to IFP1.4 (IFP), fluoresces at 708 nm (near-infrared region) when excited at 684 nm. (B) Spectral absorbance scan of purified HO1 incubated with various concentrations of heme. The Soret band maximum wavelength is indicated above the spectral peak (405 nm). (C) Fluorescent intensities of IFP1.4 and biliverdin mixtures were measured over a range of molar ratios. Biliverdin-only samples were measured at similar molar concentrations. (D) Mixtures including heme, IFP1.4, HO1, cytochrome P450 reductase (CPR), and/or NADPH were incubated at 37°C and the fluorescence (708 nm) was measured. Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation. A one-way ANOVA with Tukey's multiple comparison test was used for statistical analysis.

3.2 Construction of the sensor in bacterial cells

We next tested if the expression of both *ifp1.4* and *ho1* within a bacterial cell would lead to a heme-dependent production of fluorescence. DNA encoding epitope-tagged versions of *ifp1.4* and *ho1* were cloned into pUC19 to create a bicistronic

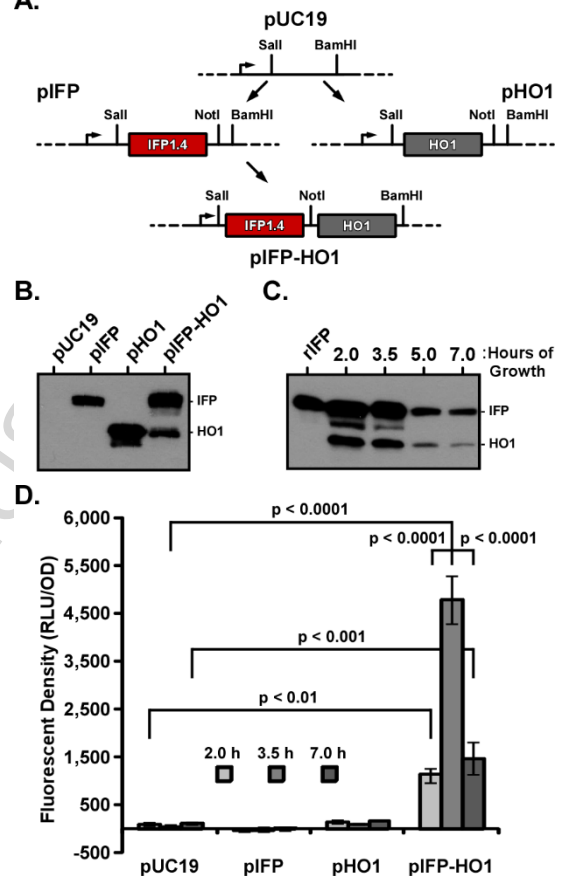
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expression system (Fig. 2A). Plasmid constructs were then transformed into an O157:H7 EHEC strain of *E. coli* (EDL933) previously demonstrated to utilize heme and hemoglobin as an iron source (Torres and Payne, 1997). Expression of both IFP and HO1 in EDL933 was confirmed by Western blot, as well as control strains transformed with plasmids harboring only a single component or none at all (Fig. 2B). A temporal analysis of the levels of these proteins during growth in culture suggests peak levels occur at about 3.5 hours, with a slow decline steadily thereafter (Fig. 2C). All strains grew equally well under these conditions (Fig. S3A). When the *E. coli* expressing these constructs were cultured in the presence of heme and the fluorescence measured, we observed that only the strain with both IFP1.4 and HO1 fluoresced to intensities above that of background (Fig. 2D). The fluorescent signal density (the fluorescent signal intensity normalized to the cell density) was greatest around late-log to early stationary phase, at roughly $t=3.5$ hours (Fig. 2D and Fig. S3B), which mirrored closely the levels of these proteins with time (Fig. 2C,D). Collectively, these data provide strong evidence that IFP and HO1, when expressed together, generate a fluorescent read-out when *E. coli* is exposed to heme.

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Figure 2. The expression of the heme sensor (IFP-HO1) A.

in E. coli. (A) *ifp1.4* and *ho1* were cloned into a pUC19 vector as shown. Western for the HA-tag from IFP1.4 and/or HO1 expressed in *E. coli* EDL933 after 3.5 hrs (B) or from 2-7 hours (C) of growth in LB at 37°C. (D) The fluorescent density (see Methods) was determined from the optical density and fluorescence for EDL933 harboring pUC19, pIFP, pHO1, and pIFP-HO1 at 2, 3.5, and 7 hrs. Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation.



3.4 *E. coli* IFP-HO1 is responsive to heme under iron-limiting conditions

We hypothesized that culturing of *E. coli* pIFP-HO1 in an iron starved environment would lead to an uptake of exogenous heme and a dose-dependent increase in the fluorescence. To test this hypothesis, we cultured *E. coli* in M9 media supplemented with 0.6% CAS amino acids (M9Cas) and 1 mM deferoxamine (DFA), an iron chelator. Under these conditions, maximal growth of *E. coli* was restricted in an iron-dependent manner with a growth promotion observed after the addition of increasing amounts of hemin or ferrous sulfate (Fig. 3A and inset). Expression of IFP and HO1 in *E. coli* cultured in iron-restricted media but supplied with heme was similar

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to the expression of these genes in *E. coli* cultured in iron-rich media with the characteristic decrease in the IFP and HO1 after 3.5h that declined slowly thereafter (Fig. 3C). Similar to results attained in iron-rich media, fluorescence was only observed from *E. coli* carrying pIFP-HO1 (Fig. 3B). A dose-dependent increase in fluorescence was observed in these cells when exposed to heme and with peak fluorescence occurring at ~3.5hrs with no fluorescence observed in the absence of heme (Fig. 3D). As previously observed, the kinetics of the fluorescence output mirrored closely the steady-state levels of the proteins, peaking at 3.5h and tailoring off thereafter. In fact, when the half-life of IFP's fluorescence was measured by inhibiting translation with kanamycin (Fig.S4A), the signal decreased seven-fold between 2 and 3.5 hours into growth (half-life of 4.6 hours and 0.65 hours, respectively), and nearly another two-fold between 3.5 hours and 5 hours (half-life of 0.37 hours) (Fig. S4B). When taken together, these data suggest that *E. coli* expressing IFP-HO1, when grown in an iron-limiting environment, are fluorescent when exposed to heme, which increases as the concentration of the iron-porphyrin is raised. It also suggests the sudden drop in signal after 3.5hrs is due to a loss in IFP-HO1, with the half-life decreasing ~7 fold after peak fluorescence, thereby signifying a rapid proteolysis of one or both components of the sensor.

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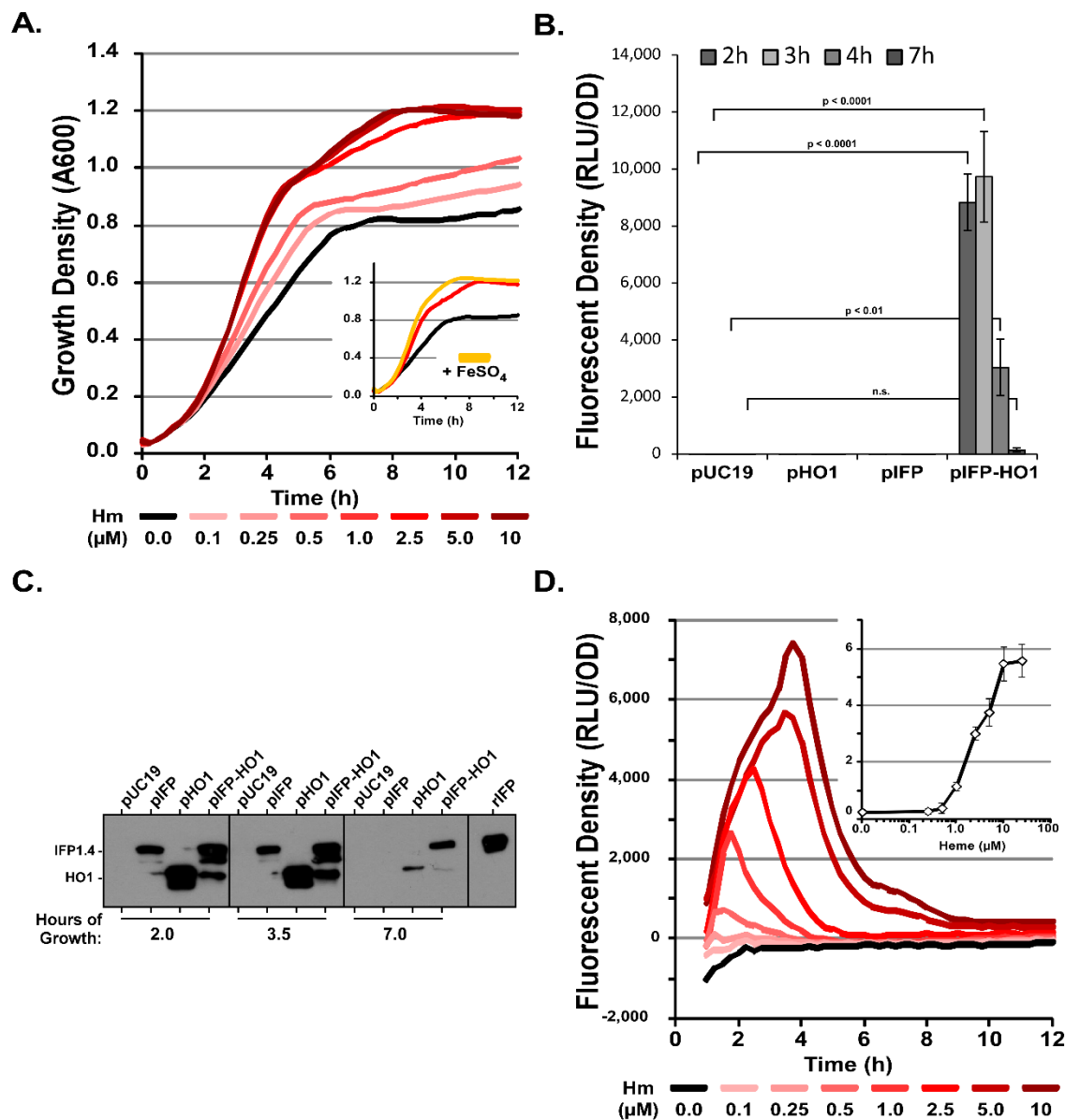


Figure 3. Fluorescence of *E. coli* (IFP-HO1) in the presence of heme. (A) *E. coli* EDL933 (IFP-HO1) was cultured in M9Cas with DFA and various concentrations of heme (Hm) and the optical density at 600 nm recorded. The inset in A shows growth in M9Cas with DFA and free iron (FeSO_4). (B) Fluorescent density of *E. coli* EDL933 cultured in iron-starved media supplemented with heme (25 μ M) at various time points (excitation at 680 nm, emission 710 nm, optical density at 600 nm). (C). Western blot detection of

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an HA-tag sequence from *E. coli* cultured in iron starved media (M9Cas with DFA) measuring abundance of IFP1.4 and HO1. (D) The fluorescent density measured from cultures shown in panel A. The inset in B plots the maximum fluorescence densities vs the concentration of heme. All data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation.

3.5 *E. coli* O157:H7 utilize *chuA* and *tonB* to transport heme

To this point, the evidence indicates that feeding heme to *E. coli* carrying a plasmid that express the IFP-HO1 sensor leads to an increase in fluorescence under iron-starved conditions. It is believed that Gram-negative bacteria utilize a protein network to transport heme across the outer and cytoplasmic membranes. *E. coli* has been used as a prototype organism to study these processes. The current model for heme uptake in *E. coli* is that the outer membrane transporter, ChuA, binds and transports heme from the extracellular environment to the periplasm, a process powered by the energy transferring complex TonB/ExhB/ExhD (Fig. 4A) (Krewulak and Vogel, 2011; Torres and Payne, 1997). It is then thought that periplasmic binding proteins then facilitate the transfer of heme to the cytoplasmic membrane ABC transporter and eventually into the cytoplasm for processing. To directly test the hypothesis that ChuA and TonB promote the intracellular transport of heme, we cultured wild-type, $\Delta chuA$, and $\Delta tonB$ strains of EHEC EDL933 carrying pIFP-HO1 under low-iron conditions and measured the levels of fluorescence from 2 to 7h (Fig. 4B). Wild-type (pIFP-HO1) *E. coli* demonstrated the characteristic plateau in fluorescence between 2 and 3h that slowly declined by 7h, as previously observed. However, both the $\Delta chuA$ and $\Delta tonB$ strains (pIFP-HO1) displayed significantly lower fluorescence at

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mid-log phase compared to wild-type, a finding that suggests less heme had entered the bacterial cytoplasm (Fig. 4B). Interestingly, an even greater reduction in fluorescence was observed when the $\Delta tonB$ strain was cultured in the presence of heme. This finding is also consistent with less heme entering the cell and may highlight the overall universal importance of TonB in the transport of ligands. Both mutant strains could be complemented by expressing a functional copy of the missing gene, which indicates this reduction is specifically attributed to the loss of ChuA or TonB. Of note, a complete kinetic analysis of the fluorescence during the growth of these strains indicated that the $\Delta chuA$ strain (pIFP-HO1) complemented for *chuA* did not undergo the characteristic time-dependent decrease in fluorescence after the 2h plateau (Fig. 4B and Fig. S5A). This was not observed for the complemented $\Delta tonB$ strain (Fig. 4B and Fig. S5B). In fact, the fluorescence stayed elevated throughout the life of the culture, meaning that the continuous and likely high expression of the ChuA receptor promotes the continuous import of heme which is then quickly converted into biliverdin by the sensor. Such a finding is advantageous if one desires to examine heme transport at later stages of cell culture. When taken as a whole, these findings support the notion that the heme-dependent increase in fluorescence in iron-starved *E. coli* expressing IFP-HO1 is due to the direct uptake and conversion of intracellular heme to biliverdin, a process that is dependent on ChuA and TonB. By extension, these results also strongly support the original model that ChuA and TonB are responsible for the transport of heme into the cell, a hypothesis to this point that was only supported by growth studies.

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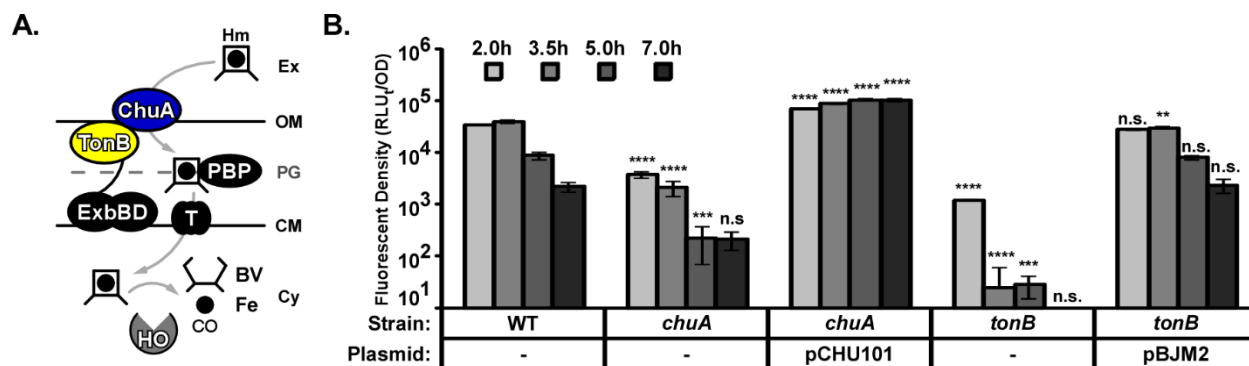


Figure 4. The fluorescence of *E. coli* (IFP-HO1) from mutants lacking proteins involved in transport – testing the model of heme transport in *E. coli*. (A) Proposed model for heme acquisition in *E. coli*: extracellular space (Ex), outer membrane (OM), peptidoglycan (PG), cytoplasmic membrane (CM), cytosol (Cy), *ChuA* (A), *TonB* (B), *ExhB* and *ExhD* (BD), periplasmic heme binding protein (P), cytoplasmic heme ABC transporter (T), heme oxygenase (HO), carbon monoxide (CO), iron (Fe), and biliverdin (BV). (B) *E. coli* EDL933 wild-type, a strain lacking *chuA* ($\Delta chuA$), or a strain lacking *tonB* ($\Delta tonB$), were cultured in the presence of heme (25 μ M) for 2-7 hrs under iron-limiting conditions at 37°C. Mutant strains were complemented using vectors *pCHU101*. Double, triple, and quadruple asterisks represent statistical significance with $p \leq 0.01$, 0.001, and 0.0001, respectively. Statistical analysis was performed using student's t-test with comparison of each mutant strain to the WT strain at the corresponding time point.

3.6 Iron drives heme biosynthesis, which is dependent on *hemE*

One interesting finding from this work was that *E. coli* (pIFP-HO1) grown in iron-starved media lacking heme yielded strong fluorescence (Fig. 4B) and that $\Delta chuA$ (and especially $\Delta tonB$), despite displaying a dramatic transport defect, still produced measurable fluorescence at the so-called fluorescence plateau (~ 2-3h). Both results suggest that a portion of the fluorescence of *E. coli* (pIFP-HO1) is due to something other than the transport of the extracellular porphyrin into the cytoplasm. We

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hypothesized that this additional fluorescence could be due to the biosynthesis of heme and subsequent breakdown and detection by the sensor. To test this hypothesis, we assessed the level of fluorescence produced by the transport mutants ($\Delta chuA$ and $\Delta tonB$), alongside a strain harboring a clean deletion in *hemE* ($\Delta hemE$). HemE encodes the enzyme uroporphyrinogen III decarboxylase, which removes four carbon dioxides from uroporphyrinogen to generate coporphyrinogen, a critical branch step in the biosynthesis of heme b (Săsarman et al., 1975). *E. coli* lacking a functional copy of *hemE* cannot synthesize heme (Săsarman et al., 1975). As such, we reasoned that a strain that lacks this enzyme, whose activity leads exclusively to the production of heme, was the most practical way to test the contribution of heme biosynthesis to the fluorescent signal observed for cells carrying the sensor. None of the mutant strains grew in the absence of iron and all received a growth boost upon the addition of an iron source, albeit with the $\Delta hemE$ strain at a lower rate (Fig. 5A) When examined for fluorescence, none of the strains yielded signal in the absence of iron due to the poor growth under these conditions (Fig. 5B). Interestingly, upon the addition of ferrous sulfate (i.e. free atomic iron), a higher level of fluorescence was observed for wild-type *E. coli*, which increased with the addition of more iron, despite the absence of heme in the culture. Even more surprisingly was that both the $\Delta chuA$ and $\Delta tonB$ strains also demonstrated this response (blue and yellow bars, respectively). Reasoning that this fluorescence was caused by an iron-dependent stimulation of the biosynthesis of heme, we tested the response of the $\Delta hemE$ strain (dark green) harboring the sensor. Indeed, the iron-dependent fluorescence observed for the wild-type and transport mutants was

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not observed for $\Delta hemE$ *E. coli*, even at the highest concentration of ferrous sulfate. Of note, the output here is fluorescent density, which normalizes the fluorescence signal to the optical density (see Methods), meaning the low values here for the $\Delta hemE$ strain are not due to poor growth. Taken together, these results suggest that the IFP-HO1 heme sensor protein reports not only on heme transport and breakdown but also on the intracellular production of heme, a useful dual-feature. That heme biosynthesis is seemingly stimulated by the uptake of free iron also highlights how important iron is for basic bacterial metabolism and the production of critical small molecule ligands such as heme.

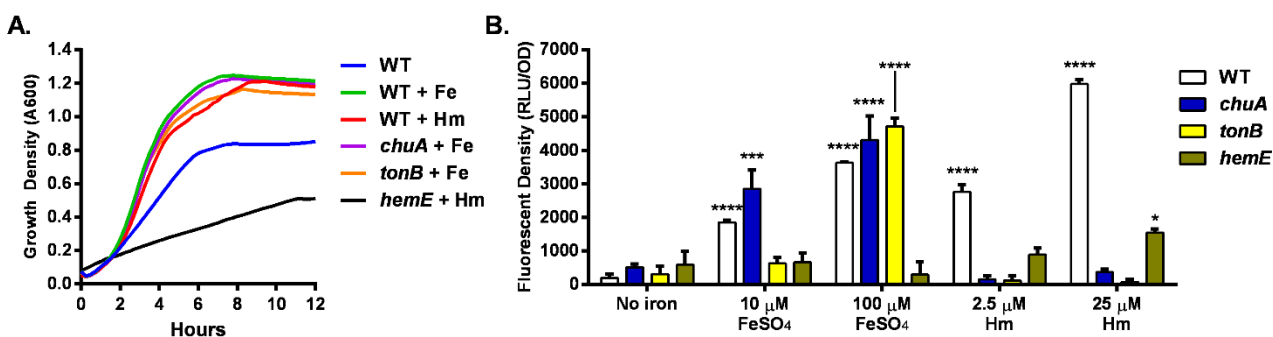


Figure 5. The use of IFP-HO1 to report on the biosynthesis of heme. (A) *E. coli* EDL933 (IFP-HO1) was cultured in the presence (heme = 25 μ M, iron = 100 μ M) or absence of iron source at 37°C for 12hrs in iron-limiting media. (B) The fluorescent densities at 3.5 hours (peak fluorescence) were measured for each strain and condition in (A). Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation. Single, triple, and quadruple asterisks represent statistical significance with $p \leq 0.05$, 0.001, and 0.0001, respectively. Statistical analysis was performed using student's t-test with comparison of each strain to its no iron data points.

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3.7 Testing the heme sensor during the infection of animals

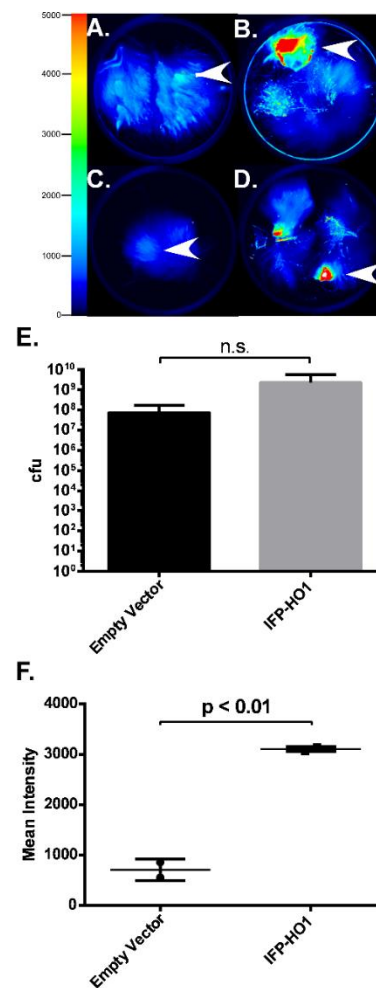
Part of the rationale for developing the IFP-HO1 heme sensor as a reporter for heme iron acquisition in bacteria is that the light emitted from IFP is in the near-infrared region. This region of emission undergoes far less absorbance from proteins and tissues in a complex biological environment such as inside a mammal (Deliolani et al., 2014; Rice and Contag, 2009; Yu et al., 2014). We wondered if IFP-HO1 could report on heme metabolism in bacteria during the live infection of a vertebrate host. For these studies, we transferred the plasmid expressing IFP-HO1 into extraintestinal *E. coli* (ExPEC) strain CP9, an invasive *E. coli* isolate that, unlike EHEC EDL933, causes a systemic infection with multi-organ involvement (Nazareth et al., 2007). We next performed two distinct types of infection, both which result in different courses of the disease. For the first study, ExPEC CP9, harboring an empty vector (control) or pIFP-HO1 was injected subcutaneously into the right or left flank of the C57/Blk mice. Preliminary work from our group suggests this route can result in a localized infection that develops into a granuloma that does not disseminate if injected bacteria are in stationary phase (data not shown). Twenty-four hours later, mice were euthanized, and each flank excised and analyzed for fluorescence. Mice infected with ExPEC containing pIFP-HO1 demonstrated fluorescence that visually was well above that observed for the empty vector control (Fig. 6A-D) with a mean measured fluorescence that was quantitatively three times higher (Fig. 6F). There was no statistical difference in the number of CFUs between the vector control and IFP-HO1 in the infected granulomas (Fig. 6E). To further evaluate the utility of the sensor, ExPEC CP9 harboring either the

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empty vector or pIFP-HO1 were injected in the intraperitoneum of mice, a route known to lead to a systemic infection (Russo et al., 1995). Twenty-four hours later, the mice were sacrificed, necropsied, and the organs excised for analysis. As shown in Fig. 7C, F, and I, the infection resulted in the dissemination of ExPEC to the spleen, kidneys, and liver, with slightly higher levels of ExPEC carrying the empty vector found in each tissue. Interestingly, each of the tissues showed some degree of autofluorescence at 708nm, as assessed by the degree of light observed in tissues harboring ExPEC carrying only the vector (Fig. 7A, D, and G). However, and for each tissue measured, mice infected with ExPEC bearing IFP-HO1 showed a marked increase in the fluorescence intensity, with the greatest difference (~ 5-fold) observed in the spleen (Fig. 7B, E, and H). When taken as a whole, these findings indicate that *E. coli* expressing IFP and HO1, whether localized or disseminated to major organs during an infection, produces a fluorescent signal well above background that can be both visualized and quantified. The results also suggest, by virtue of the properties of this heme sensor demonstrated herein, that this pathogenic strain of *E. coli* undergoes substantial heme turnover during infection, a process that may represent either the import or the synthesis of heme (or both). Future studies will delineate the molecular aspects of this observation.

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Figure 6. Subcutaneous infection of mice with ExPEC (IFP-HO1). Mice were subcutaneously injected with 10^8 cfu of CP9 carrying the empty vector (A,C) or IFP-HO1 (B,D), the mice sacrificed at 24hrs, granulomas containing bacteria excised and subjected to imaging with a Li-Cor Odyssey to collect fluorescence at ~708 nm. Arrows indicate the site of infection/granuloma. The color bar represents the mean fluorescent intensity. (E) Colony forming units (cfus) were determined by plating the granulomas on LB-agar^{Amp} (n.s. = no statistical significance). (F) The mean fluorescent intensity of granulomas. The data represent the mean from two separate mouse injections and error bars represent the standard deviation. Statistical analysis was performed using Student's t-test.



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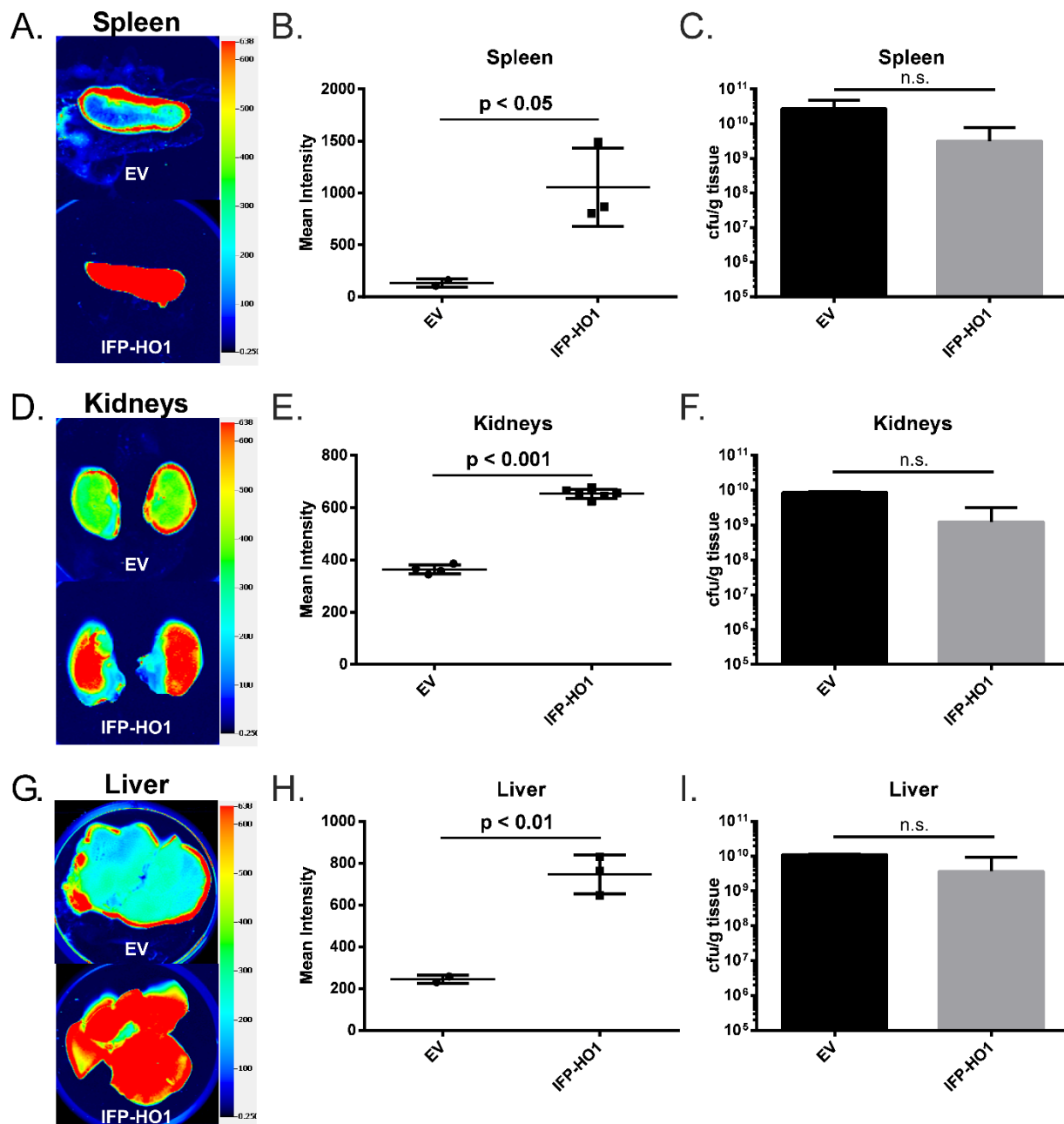


Figure 7. Intraperitoneal infection of mice with ExPEC (IFP-HO1). ExPEC strain CP9 (1×10^8 cfus) harboring either the empty vector or IFP-HO1 were injected into peritoneum of mice and 24 hrs later the animals were necropsied, the organs (spleen, kidney, and liver) excised, imaged (A, D, and G), the fluorescence emitted at 708 nm quantified (B, E, and H), and CFUs from each tissue (C, F, and I) determined by plating on LB-agar^{Amp}. EV = empty vector. The data points represent the mean of three

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separate mouse infections and the error bars represents the standard deviation. No significance is annotated n.s. Statistical analysis was performed using a Student's t-test.

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4. Discussion

This study assessed the utility of coupling the production of biliverdin by HO1, a heme oxygenase from humans, to fluorescence produced by IFP, a bacterial phytochrome protein, to assess the state of heme in bacterial pathogens under a range of environmental conditions. Our data demonstrate (i) that purified HO1 and IFP, when mixed with heme, produces fluorescence in the near-infrared region that requires all 3 factors, (ii) that this outcome can also be reproduced in living bacterial cells expressing both IFP and HO1 (heme sensor), including diarrheagenic (EHEC) and invasive (ExPEC) forms of *E. coli*, (iii) that one can approximate the amount of biliverdin made (and thus heme turned over) if the optical density and fluorescence of the culture are known, (iv) that the sensor reports on the uptake (and breakdown of heme) in a dose and time-dependent manner, and can be used to assess the contribution of transporters to the uptake process, (v) that in the absence of heme, but in the presence of iron, the sensor also reports on heme biosynthesis, and can be used to assess the role of biosynthetic enzymes in the process, (vi) that the fluorescence will decay over time, which likely is due to the degradation of the sensor, but that this process can be countered if a heme receptor such as ChuA is continuously expressed, and (vii) that the sensor can report on heme homeostasis of a localized and invasive infection, thereby expanding its versatility from cells to vertebrate hosts.

Heme acquisition systems are required for bacterial virulence and can likely be targeted for the development of antimicrobial strategies (Furci et al., 2007; Owens et al., 2013). Classically, key genes involved in heme uptake have been identified and

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characterized through genetic techniques (using methods of gene inactivation and complementation), bioinformatic analyses (locating homologous genes/proteins, identifying possible regulatory elements) and/or by biochemical methods (identifying heme binding proteins through spectral analysis). Yet many pathogens utilize redundant heme uptake systems, making identifying the role of these genes and proteins in cellular functions and infection models difficult and confounding. Furthermore, current methods to assess heme steady-state levels use chemical means that are destructive to cells or systems, and there are currently no methods to assess the status of heme in live animals (Sinclair et al., 2001). To address this issue in the field, we wished to develop a non-invasive way to measure heme levels in complex biological environments. Having a quantitative output in assays for heme utilization experiments would help in identifying redundant systems, could be used to identify new genes involved in heme homeostasis (or molecules that inhibit them), and allow one to follow heme-based processes in real time in cells or animal model systems. The near-infrared fluorescent protein (IFP1.4) was developed in 2009 by engineering a bacteriophytochrome (photochromatic histidine kinase) from *Dienococcus radiodurans* (Shu et al., 2009; Weissleder and Ntziachristos, 2003). Unique to this fluorescent protein, IFP1.4 must bind to a chromophore in order to fluoresce in the near-infrared region, as neither the protein nor the chromophore is independently fluorescent to a significant extent. The utility of this fluorescent probe sparked interest for two main reasons. First, it fluoresces in a spectral range that has minimal noise and absorbance, thereby making it optimal for imaging through animal tissue compared to visible range

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fluorescent proteins. Unlike other visible light, fluorescence in the red to near infrared range (650 – 900 nm) can penetrate animal tissue with little to no scattering of light, leading to higher signal to noise ratios for greater sensitivity. Hemoglobin and water primarily absorb visible light within animal tissue, while lipids are strong absorbers of infrared light, creating a window within the red to near infrared range with the lowest absorption coefficients in animal tissue (Jöbsis, 1977; Weissleder and Ntziachristos, 2003). Second, the chromophore is the immediate product of heme degradation in humans, biliverdin IX α , and thus can be used in bacteria that utilize heme to report on the production of this product (Jöbsis, 1977; Weissleder and Ntziachristos, 2003).

We hypothesized that expression of IFP1.4 would lead to a direct increase in the fluorescence of bacteria that were actively breaking down heme as an iron-source. The enzyme HO1 was identified as a heme oxygenase that produced the α -isoform of biliverdin, the form that binds IFP (Shu et al., 2009; Wilks et al., 1995). Bacterial heme oxygenases, however, can also produce other isoforms of biliverdin, including β -, γ -, and δ -isoforms (Fujii et al., 2004; Lee et al., 2014; Runyen-Janecky, 2013). Based on structural information of the bacteriophytochrome and biliverdin interaction, isoforms of biliverdin other than the α -isoform would not be capable of binding to IFP1.4 and therefore could not be used for the purposes here. No information has been published on the *E. coli* ChuS regarding heme oxygenase regiospecificity, yet the protein shares a great deal of structural homology and sequence identity (41%) with *Pseudomonas aeruginosa* PhuS heme oxygenase. Data regarding the regiospecificity of heme breakdown by PhuS suggests that ChuS may produce the β -isoform of biliverdin (Lee et

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al., 2014; Suits et al., 2006). This is consistent with our data since *E. coli* grown on heme as an iron source while expressing only IFP1.4 did not lead to an increase in the fluorescence of cells (Fig. 3B). The addition of HO1 to the reporter plasmid led to a robust increase in the near-infrared fluorescence in *E. coli*, which was consistent with what was also observed biochemically. Both IFP1.4 and HO1 are needed to generate fluorescence within *E. coli*. These data mean that *E. coli* does not produce biliverdin Ix α , that isoforms of biliverdin that *E. coli* does produce do not lead to fluorescence with IFP1.4, and that in order to utilize IFP1.4 as a heme sensor (in this particular case), heme catabolism must be redirected to form biliverdin Ix α by HO1. This thus formed the basis of using the IFP-HO1 pair.

It is known that depletion of intracellular heme by expression of a heme “sink” leads to the over production of heme via biosynthesis to rescue the deficiency (Verderber et al., 1997; Woodard and Dailey, 1995). The expression of HO1 in *E. coli* certainly fills the role of a heme sink, yet it is unlike the proteins used in previous studies, as it degrades heme compared to sequestering it. The fluorescence increase after supplying iron to the cultures is therefore likely to be representative of a large boost in heme production. Though it has not been determined how heme or iron can regulate heme biosynthesis in *E. coli*, it has been demonstrated that heme is an inhibitor of biosynthesis (Verderber et al., 1997; Woodard and Dailey, 1995). A novel finding here is that our data suggest that iron itself promotes heme biosynthesis, possibly as a signal that enough iron is present in the cell to be incorporated into the newly synthesized porphyrin. It is not known if the increase in intracellular iron from

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exogenous heme uptake and degradation can also initiate heme biosynthesis. The process of degrading heme only to synthesize it seems physiologically senseless and energetically wasteful, but our data does suggest that the biosynthesis of heme is important for the initiation or maintenance of heme uptake, since neither growth nor the fluorescent signal is restored when heme is given as an iron source to the biosynthetic mutant *hemeE*.

When considering the use of reporters in cells of any kind, it is necessary to consider the effect their presence may have on the biology of the topic being studied. Of note, an RT-qPCR reaction designed to probe the levels of mRNA from bacterial genes involved in heme uptake (*chuA*) and biosynthesis (*hemA* and *hemE*) yielded no significant difference in the expression of these genes between *E. coli* O157:H7 EDL933 cells carrying either the empty vector (pUC19) or the IFP-HO1 construct (pIFP-HO1) in cells grown under either iron-rich (LB) or iron-deficient (M9 + 0.6% CasAA) conditions (Fig. S6A,B). These data suggest that, at least at the level of the expression of a major heme transporter and two heme biosynthesis genes, the sensor does not alter the post-transcriptional regulation of these systems. Another noteworthy finding of our study is that heme seems to stabilize the sensor. A rapid decay of the fluorescent signal occurs about 4 hours in cultures of *E. coli* under conditions where signal is generated from heme uptake as well as heme biosynthesis. Several possibilities could explain this decay in fluorescence. A decrease in the abundance of the reporting system, both IFP1.4 and HO1, does appear to reduce the maximum fluorescent potential of *E. coli*, and there is solid evidence presented here that the sensor has a

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reduced half-life after ~ 3 hours of growth. There are two possible explanations for this. The first is that there is an increase in protease activity later in growth that leads to a rapid breakdown of both IFP and HO1. Given the rapid decay of IFP-biliverdin, it is possible that large amounts of IFP and HO1 are lost during sample preparation for protein detection, or that the sensor is degraded in live cells. Second, previous work that described the use of the same expression system concluded that protein abundance decreased on a per cell basis with increasing time when *E. coli* transitions from a log-growth phase to a stationary growth phase (Woodard and Dailey, 1995). It seems that in some way the sensor undergoes proteolysis later in cultures but that this is countered by being exposed to heme, since steady-state levels of both proteins increased with increasing amounts of heme. Furthermore, the fluorescence is stabilized when the $\Delta chuA$ strain is complemented with a plasmid expressing functional *chuA*. The pCHU101 plasmid carries a copy of *chuA* on a pACYC184 backbone, and remains under Fur regulation. A low copy number plasmid, the pCHU101 vector still supplies the *E. coli* with many copies of *chuA*, leading to a much higher expression of *chuA* than wild-type (Torres and Payne, 1997). It would seem that the amount of heme transported and sensor stability are directly linked, leading to sustained levels of fluorescence (Fig. 4B) and increased protein abundance (Fig. S5D) from the complemented *chuA* mutant. This can be an advantageous use of the sensor if continuous expression of the system is needed to answer the particular question being posed by the investigator. Regardless, by simply taking a kinetic approach to measuring the fluorescence under whatever conditions are being assessed, especially at peak expression of the sensor,

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concerns about the sensor's stability can be alleviated. Future work will focus on developing "upgrades" of IFP that emit more light, as well as determining if the use of protease inhibitors can stabilize the sensor.

The utility of IFP-HO1 to examine fundamental biological processes related to heme metabolism and homeostasis is realized in the work presented here as well as what is possible in the future. As such, the use of the sensor allowed us to more directly test a long-standing model that ChuA and TonB participate in heme uptake in *E. coli*, and work presented here allows one to integrate the sensor into a clear picture of how it reports on heme intake and biosynthesis (Fig. 8). An increasing number of bacterial species, both commensal and pathogenic in origin, are being characterized as having heme acquisition pathways and the importance of heme acquisition has only recently come to light in the last ten years or so (Nobles and Maresso, 2011; Runyen-Janecky, 2013). Key proteins and enzymes were identified through genetic, biochemical, and bioinformatic methods, yet the identification of these key players has raised many questions than answers. How are heme acquisition systems regulated? How is heme biosynthesis regulated? How does *E. coli* manage and react to excess heme and heme toxicity and what is the relationship between iron and heme uptake? In combination with traditional molecular biology techniques, the co-expression of *ifp1.4* and *ho1* can help to begin to answer these questions. As this system was developed on a plasmid, it could be transferred to other expression vectors or incorporated into the genome. The generation of a mutant library in strains expressing the sensor, and then screening for loss of fluorescence, can identify genes involved in heme uptake, regulation, or

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biosynthesis. Likewise, utilizing a small molecule library could identify new pharmaceuticals which may be able to compete with or inhibit heme uptake. But perhaps the most exciting aspect of this work are the promising results the sensor displays here is shown here it has shown in mice. To our knowledge, there has only been limited work demonstrating the genes involved in heme biology are expressed in bacteria during infection (Reniere and Skaar, 2008; Stauff and Skaar, 2009). With this sensor, and the newer version of IFP that has been made even brighter (Yu et al., 2014), it may be possible to not only track infections in real-time due to the tissue penetrating power of near-infrared light, but to also determine the heme requirements for pathogens during different stages of infections and in different areas of the body.

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5. Conclusions

This study develops and proves a novel biosensor for studying heme metabolism in pathogenic *E. coli* in real-time using fluorescence, both *in vitro* and *in vivo*. Future studies will be directed towards utilizing the biosensor to monitor bacterial heme metabolism in live mice, monitoring genes involved in heme metabolism, and screening for small molecules which disrupt heme metabolism.

Acknowledgments

This work was supported by AI069697 and AI116497 from National Institutes of Health, a Dunn Collaborative Research Foundation Grant, and seed funds from Baylor College of Medicine to A.W.M. We thank Dr. James Johnson (University of Minnesota) for ExPEC strain CP9 and Dr. Alfredo Torres (University of Texas Medical Branch) for supplying *E. coli* O157:H7 and plasmids. Author contributions: CLN, JRC, and AWM designed the experiments, analyzed the data, and wrote the paper. CLN and JRC performed the experiments. The authors declare no conflict of interest.

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Figure Legends

Figure 1 – The construction of a heme biosensor using HO1 and IFP1.4. (A) Heme (Hm) can be oxidatively degraded by HO1 to produce the products of carbon monoxide (CO), biliverdin IX α (BV), and iron (Fe). Biliverdin, when bound to IFP1.4 (IFP), fluoresces at 708 nm (near-infrared region) when excited at 684 nm. (B) Spectral absorbance scan of purified HO1 incubated with various concentrations of heme. The Soret band maximum wavelength is indicated above the spectral peak (405 nm). (C) Fluorescent intensities of IFP1.4 and biliverdin mixtures were measured over a range of molar ratios. Biliverdin-only samples were measured at similar molar concentrations. (D) Mixtures including heme, IFP1.4, HO1, cytochrome P450 reductase (CPR), and/or NADPH were incubated at 37°C and the fluorescence (708 nm) was measured. Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation. A one-way ANOVA with Tukey's multiple comparison test was used for statistical analysis.

Figure 2 – The expression of the heme sensor (IFP-HO1) in *E. coli*. (A) *ifp1.4* and *ho1* were cloned into a pUC19 vector as shown. Western for the HA-tag from IFP1.4 and/or HO1 expressed in *E. coli* EDL933 after 3.5 hrs (B) or from 2-7 hours (C) of growth in LB at 37°C. (D) The fluorescent density (see Methods) was determined from the optical density and fluorescence for EDL933 harboring pUC19, pIFP, pHO1, and pIFP-HO1 at 2, 3.5, and 7 hrs. Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation.

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Figure 3 – Fluorescence of *E. coli* (IFP-HO1) in the presence of heme. (A) *E. coli* EDL933 (IFP-HO1) was cultured in M9Cas with DFA and various concentrations of heme (Hm) and the optical density at 600 nm recorded. The inset in A shows growth in M9Cas with DFA and free iron (FeSO_4). (B) Fluorescent density of *E. coli* EDL933 cultured in iron-starved media supplemented with heme (25 μM) at various time points (excitation at 680 nm, emission 710 nm, optical density at 600 nm). (C). Western blot detection of an HA-tag sequence from *E. coli* cultured in iron starved media (M9Cas with DFA) measuring abundance of IFP1.4 and HO1. (D) The fluorescent density measured from cultures shown in panel A. The inset in B plots the maximum fluorescence densities vs the concentration of heme. All data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation.

Figure 4 – The fluorescence of *E. coli* (IFP-HO1) from mutants lacking proteins involved in transport – testing the model of heme transport. (A) Proposed model for heme acquisition in *E. coli*: extracellular space (Ex), outer membrane (OM), peptidoglycan (PG), cytoplasmic membrane (CM), cytosol (Cy), ChuA (A), TonB (B), ExhB and ExhD (BD), periplasmic heme binding protein (P), cytoplasmic heme ABC transporter (T), heme oxygenase (HO), carbon monoxide (CO), iron (Fe), and biliverdin (BV). (B) *E. coli* EDL933 wild-type, a strain lacking *chuA* ($\Delta chuA$), or a strain lacking *tonB* ($\Delta tonB$), were cultured in the presence of heme (25 μM) for 2-7 hrs under iron-limiting conditions at

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37°C. Mutant strains were complemented using vectors pCHU101 (carrying *chuA*) or pBJM2 (carrying *tonB*). Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation. Data were analyzed using one-way ANOVA with Tukey's multiple comparison. No significance is represented by n.s. Double, triple, and quadruple asterisks represent statistical significance with $p \leq 0.01$, 0.001 , 0.0001 , respectively.

Figure 5 – The use of IFP-HO1 to report on the biosynthesis of heme. (A) *E. coli* EDL933 (IFP-HO1) was cultured in the presence (heme = 25 μ M, iron = 100 μ M) or absence of iron source at 37°C for 12hrs in iron-limiting media. (B) The fluorescent densities at 3.5 hours (peak fluorescence) were measured for each strain and condition in (A). Data points represent the mean of experiments performed in triplicate and the error bars represent the standard deviation. Single, triple, and quadruple asterisks represent statistical significance with $p \leq 0.05$, 0.001 , and 0.0001 , respectively. Statistical analysis was performed using student's t-test with comparison of each strain to its no iron data points. Double and quadruple asterisks represent statistical significance with $p \leq 0.01$ and 0.0001 , respectively.

Figure 6 – Subcutaneous infection of mice with ExPEC (IFP-HO1). Mice were subcutaneously injected with 10^8 cfu of CP9 carrying the empty vector (A,C) or IFP-HO1 (B,D), the mice sacrificed at 24hrs, granulomas containing bacteria excised and subjected to imaging with a Li-Cor Odyssey to collect fluorescence at ~708 nm. Arrows

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indicate the site of infection/granuloma. The color bar represents the mean fluorescent intensity. (E) Colony forming units (cfus) were determined by plating the granulomas on LB-agar^{Amp} (n.s. = no statistical significance). (F) The mean fluorescent intensity of granulomas. The data represent the mean from two separate mouse injections and error bars represent the standard deviation. Statistical analysis was performed using Student's t-test.

Figure 7 – Intraperitoneal infection of mice with ExPEC (IFP-HO1). ExPEC strain CP9 (1×10^8 cfus) harboring either the empty vector or IFP-HO1 were injected into peritoneum of mice and 24 hrs later the animals were necropsied, the organs (spleen, kidney, and liver) excised, imaged (A, D, and G), the fluorescence emitted at 708 nm quantified (B, E, and H), and CFUs from each tissue (C, F, and I) determined by plating on LB-agar^{Amp}. EV = empty vector. The data points represent the mean of three separate mouse infections and the error bars represents the standard deviation. No significance is annotated n.s. Statistical analysis was performed using a Student's t-test.

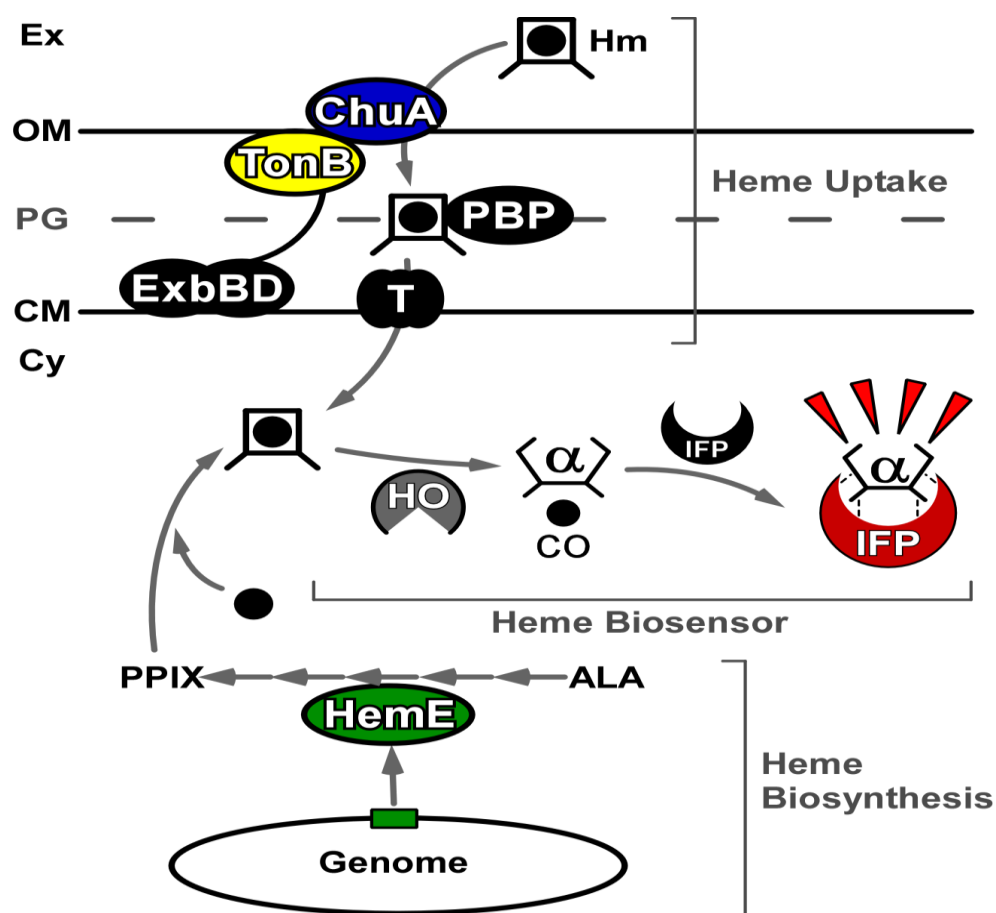
Figure 8 – A near-infrared fluorescent heme sensor to report on bacterial heme metabolism. Heme is transported via ChuA or synthesized via a biosynthetic system that includes HemE in Gram-negative bacteria. In the presence of IFP-HO1, intracellular heme is degraded by the heme oxygenase and the biliverdin product is bound by IFP1.4, which emits near-infrared light at 708 nm. Abbreviations: ChuA (A), TonB (B), ExhB and ExhD (BD), periplasmic heme binding protein (P), cytoplasmic heme ABC

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transporter (T), HO1 (HO), IFP1.4 (IFP), carbon monoxide (CO), 5-aminolevulinate (ALA), protoporphyrin IX (PPIX), extracellular space (Ex), outer membrane (OM), peptidoglycan (PG), cytoplasmic membrane (CM), and cytosol (Cy).

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Graphical abstract



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Highlights

- Infrared Fluorescent Protein and Heme Oxygenase I can be coupled to generate a heme biosensor.
- The heme biosensor can monitor intracellular heme in real-time using fluorescence.
- The heme biosensor detects both heme uptake and heme biosynthesis.
- The heme biosensor verifies the current model of heme metabolism in *E. coli*.
- The heme biosensor is viable during infection using a mouse model.

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